ANNALS OF THE NEW YORK ACADEMY OF SCIENCES
Volume 63, Art. 6, Pages 1033–1462

Editor
Roy Waldo Miner

CANCER CYTOLOGY AND CYTOCHEMISTRY

BY

M. J. Kopac (Conference Chairman), Ayre, J. E., Bader, G. M., Biesele, J. J., Blumenthal, E. D., Bostrom, R. C., Cowdry, E. V., Crozier, R., Dalton, A. J., Day, E., Durfee, G. R., Duryee, W. R., Erickson, C. C., Falk, E. A., Felix, M. D., Fitzgerald, P. J., Foot, N. C., Foraker, A. G., Godwin, J. T., Graham, J. B., Graham, R. M., Grand, C. G., Grey, C. E., Gusberg, S. B., Hamonic, M. J., Hecht, E. L., Herbut, P. A., Hill, J. C., Holmquist, D. G., Hsu, T. C., Klemperer, P., Koprowska, I., Koprowski, H., Koss, L. G., Lapid, L. S., Long, M. E., Luse, S. A., MacCardle, R. C., Meigs, J. V., Mellors, R. C., Moorhead, P. S., Mottram, F. C., Nieburgs, H. E., Papanicolaou, G. N., Reagan, J. W., Rubin, C. E., Taylor, Jr., H. C., Tolles, W. E., Tovell, H. M. M.

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NEW YORK
PUBLISHED BY THE ACADEMY
March 30, 1956

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Volume 63, Art. 6, Pages 1033–1462
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Editor
Roy Waldo Miner

CANCER CYTOLOGY AND CYTOCHEMISTRY*

Conference Chairman and Consulting Editor
M. J. Kopac

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* This series of papers is the result of a conference on *Cancer Cytology and Cytochemistry* held and supported njointly by The New York Academy of Sciences, Section of Biology, and The Damon Runyon Memorial Fund Cancer Research, Inc., New York, N. Y., May 26, 27, and 28, 1955.

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INTRODUCTORY REMARKS

By Hilary Koprowski

Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

In the short time of my tenure as Chairman of the Section of Biology of the New York Academy of Sciences, I have had the honor of opening several con-erences in the field of biology. In some cases, I did not have the faintest idea what the conference was about. On several occasions I had only a vague notion, and in a few—very few—instances I was familiar with the general heme, although much less so than the participants.

The subject of the conference on which this monograph is based evokes quite a different attitude on my part. Since my wife is an exfoliative cytologist, if you will pardon this term, I felt related to the conference—by marriage, of course. In the course of many years I have become conditioned to such terms as cancer cells, histiocytes, hyperchromatism, nuclear-cytoplasmic ratio, and dyskaryosis. These terms, as well as the names of Papanicolaou, Meigs, Emerson Day, Reagan, and many others became part of my life on evenings and week ends, when my wife and I could see each other. My ear is trained now and, when I recognized these terms in the titles of your papers and the abstracts, they sounded so familiar to me that I almost felt like saying, "Hello, histiocytes, I know who you are, but I do not know what I see when I look at you."

Many scientists collaborating in this monograph are still young, some are middle-aged, none are old, since, according to Swift, "Every man desires to ive long, but no man would be old." The younger workers should remember hat they did not come "to give," in the words of Oscar Wilde, "to those who are older than themselves the full benefits of their inexperience." The middle-ged should keep in mind that "to profit from good advice requires more wisdom than to give it" (J. C. Collins).

I had planned to end these introductory remarks by saying that I hoped the data presented would so impress you that—regardless of sex—you would all ave smears taken and examined by exfoliative cytology to prove to yourselves now healthy you are. I am starting you off with the following quotation from Berkeley: "Upon the whole, I am inclined to think that the far greater part, f not all, of those difficulties which have hitherto amused philosophers, and locked up the way to knowledge, are entirely owing to ourselves. That we ave first raised a dust, and then complain we cannot see."

Part I. General Problems in Cancer Cytology

THE CONFERENCE ON CANCER CYTOLOGY AND CYTOCHEMISTRY: ITS AIMS AND RESULTS

By M. J. Kopac
Department of Biology, Graduate School of Arts and Science, New York University, New York, N. Y.

Cancer cytology, in present-day usage, generally means exfoliative cytology as applied to the diagnosis of certain neoplasms. In the conference on which this monograph is based, cancer cytology was used in its widest sense; that is, as a consideration of the morphology, physiology, and cytochemistry of neoplastic cells. The basic questions are: What are cancer cells? What are precancer cells? What are their distinguishing characteristics? What are the factors that divert normal cellular growth and differentiation into neoplastic growth? The conference was organized in the hope that some answers to these questions might be forthcoming. The topics covered in six formal sessions included general problems in cancer cytology; morphologic imprints of malignancy on cells; specialized developments in cytochemistry; cytology of early cancer; and perspectives in cancer cytology.

One aim of the conference was to establish a balance between experimental, theoretical, basic, and applied cytology and cytochemistry. An attempt was also made to achieve a balance between the diagnostic applications and other applications of cancer cytology that include endocrinology, viruses, tissue cultures, and estimations of radiosensitivity in cells. Furthermore, our attention was focused on the cytology of early cancer and on evidence that might indicate the existence of precancer cells. The integration of basic and applied cytology thereby brought the attention of all specialists assembled at the conference to the fundamental problems dealing with cancer cells.

Cytochemistry, either directly or indirectly, plays an important part in the study of all cells. Accordingly, it seemed proper to include the newer aspects of this branch of study in the monograph. Emphasis was placed on techniques, ideas, and the more recent instrumentation devised for these approaches. All these facets have increased our knowledge of cell structures and their properties and, moreover, they are potentially capable of increasing still further our explorations into these fields. Included in this monograph are such topics as the application of soft X rays, the interference microscope, the ultraviolet color translating microscope, the cytoanalyzer, and topological cytochemistry. There are frequent references to the applications of cytochemical methods, especially those concerned with nucleic acids.

At the conference, an evening session was devoted to an informal round table discussion on precancer cells. Doctor James W. Reagan, as moderator, elicited a fruitful discussion on "A New Look at the Precancer Cell." The panel for this interesting meeting included: Doctors Ayre, Biesele, Duryee, Erickson, J. B. Graham, and Koss. Perhaps the most important result of this round table, aside from expressions of biologic, cytologic, pathologic, and clinical points of view were the discussions of problems of precancer as faced by pathologists and clinicians.

Obviously, the reality of the precancer cell is as difficult to establish as are answers to questions concerning carcinoma *in situ*. Again, the basic issues are questions of detailed knowledge and perspectives. The cytologist sees certain changes in cells that are unmistakable departures from normality. These changes are frequently evident in the cells of invasive carcinomas. It is yet to be established whether the morphologic, cytochemical, or other changes seen in cells before suspected lesions become invasive are the ones that would lead inevitably to an invasive carcinoma. Until such problems are settled, it is almost impossible to define the properties, or even to justify the probable existence, of precancer cells. In fact, one might even extend this to the problems of defining the properties of cancer cells!

The precancer cell, if it exists, can be considered as an aberrant development in a cell and, if this new situation were to continue and be propagated, it might lead to carcinoma. Transitional stages in normal cellular biology are nothing new. Perhaps the best known are the prophase and other stages associated with mitosis. Without full knowledge of the cells in question, an investigator might overlook the prophase or else its significance might be misunderstood. Another well-known transitional stage is the female gamete following activation by a male gamete. In most instances, one can predict with considerable precision the subsequent series of events. That this can be done is made possible by information already available. Perhaps when we know as much about cancer cells, we may also be able to offer predictions that are both precise and reliable.

A thorough search should be made for experimental material that will shed more light on precancer cells and on carcinoma *in situ*. In this respect, the studies on the renal adenocarcinoma of the frog as reported in this monograph by Duryee[1] should be helpful toward elucidating the status of precancer cells. Likewise, further attention should be focused on carcinoma *in situ*—since this lesion might be considered as, probably, one of the primary steps in the hierarchy of cell populations that leads to invasive and thereby malignant carcinoma. The carcinoma *in situ* is also, perhaps, the smallest and most confined lesion that can be attacked from cellular points of view. It represents an incipient integration within a finite population of cells that behaves atypically. In this connection, it is clear that a single cell is not cancer; but when does a population of cells become cancer?

One of the great strides in recent years has emerged from the study of exfoliated cells and the application of these studies to diagnosis of certain neoplasms. In the first place, cells available for diagnostic study are actually taken, so to speak, out of context (spontaneously or otherwise), both in reference to space and time. Aside from the recognition that deterioration occurs when cells become separated from their normal sites, there are frequently other difficulties. The identification and interpretation of exfoliated cells is as difficult as the identification of a phrase taken out of context from an essay. At best, the exfoliated cells present no more clues to a situation than perhaps a reasonably complete sentence. Yet, on the basis of this meager information, one is asked to identify not only the equivalent of an essay but perhaps eve the author! Obviously, if one knew either the essay from which the phrase o sentence was taken or the style of the author, there might be a fairly goo chance of identifying the collection of words and placing them into prope context.

The problems facing cytologists are no less simple. The identification of cell or a group of cells must require expert knowledge of cell structures, whil the interpretations must be based on information about the lesions from whic the cells came. It is inconceivable that sound applications of exfoliativ cytology could be made without relevant data on changes that may occur i cells following various exciting influences. Furthermore, without the back ground knowledge of the lesions, it would be difficult to interpret the cells an thereby place them in correct context.

From the point of view of the cytologist, it is essential to learn more abou cells, especially about nuclear structures. To emphasize this point, a discussio is included on morphologic imprints of malignancy on cells. In this discussio much of the important information concerning mitosis, chromosomes, nucleol cytochemical changes, and fine structures relevant to cancer cells is reviewed It is hoped that a further study of these aspects will lead to a more precise dis tinction between normal and malignant cells, and the possible transitiona forms between the two. Furthermore, it is to be expected that as more con stant attributes of malignancy can be recognized in cells, methods and instru mentation will be devised not only for further study of such attributes but als as an aid in finding cells in mixed populations with the required characterisics Cell structure and function go hand in hand and, whenever possible, the tw should be studied simultaneously.

Much of the research on cells requires highly complicated procedures an expensive instrumentation. There is no need to justify such approaches, i new information about normal and neoplastic cells can be elicited. It is i such research that the basic scientists may offer important and lasting con tributions. Once a characteristic feature of a cell has been described and it significance elucidated, it becomes a problem of biological engineering to adap the necessary procedures for routine applications.

Even though the approach to cancer in this monograph has been focuse largely on cells, one must not lose sight of the other problems of equal impor tance. These are the populations of cells that make up the lesion, either *in sit* or invasive. The requisite knowledge now is so extensive that the activ participation and collaboration of specialists must be enlisted. As expresse by Meigs,[2] the policy of the Inter-Society Cytology Council stresses the neces sity of active collaboration of cytologists, pathologists, and clinicians. Thi is a healthy situation, if based on teamwork. These groups, however, shoul be augmented by adding a fourth echelon—the basic scientists—thus establish ing a tetrahedral attack on the problems of cancer cytology and cytochemistry

Cancer cytology probably had its origin in the hands of Johannes Müller who

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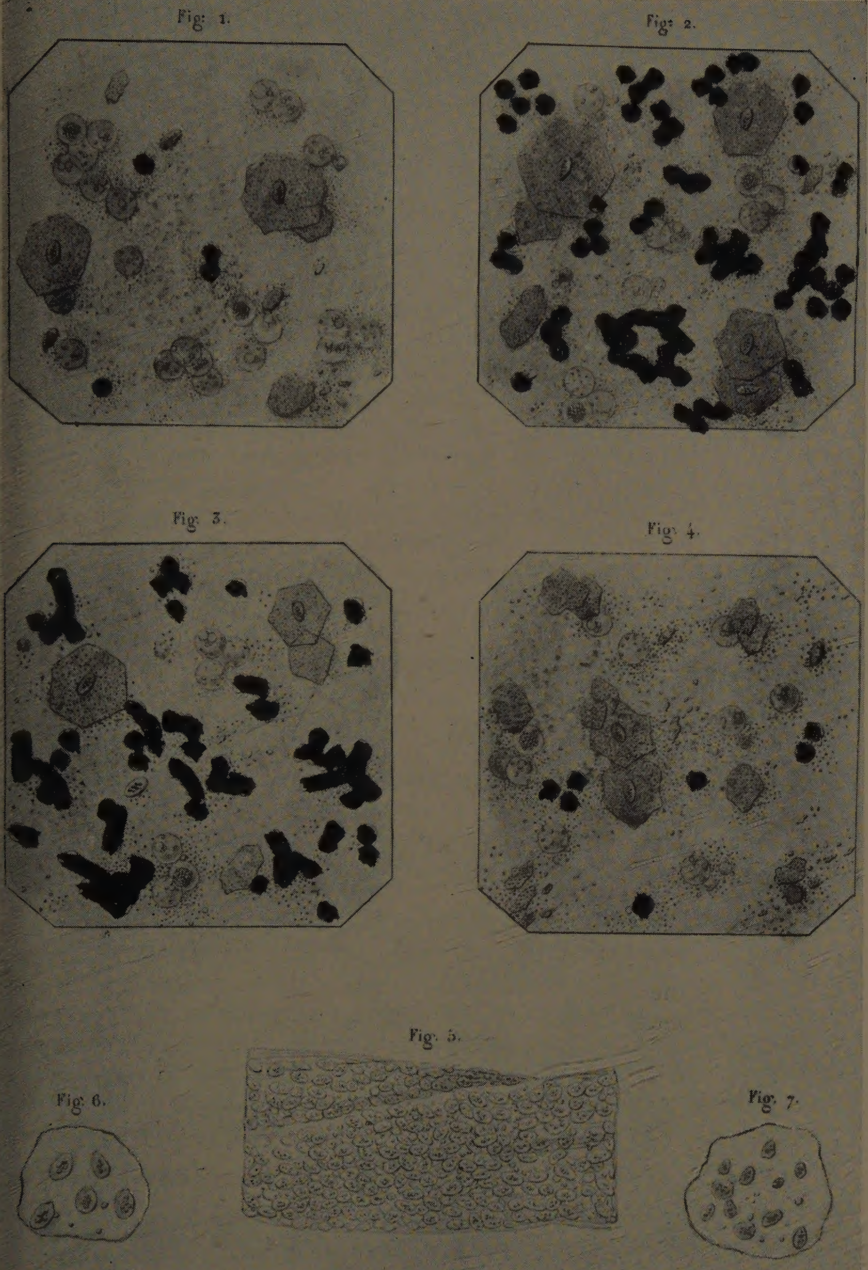
PLATE 1. Reproduction of Plate XII from Pouchet's atlas.[5] The figures illustrate the epithelial cells ob served in vaginal secretions obtained during menstruation. FIGURE 5 shows a fragment of decidua shed spon taneously 10 days after the end of the menstrual period, while FIGURES 6 and 7 show somewhat higher magnifi cations of the deciduum, which was slightly compressed. Original size of FIGURES 1 to 4 was 60 mm. across (Courtesy of the New York Academy of Medicine Library.)



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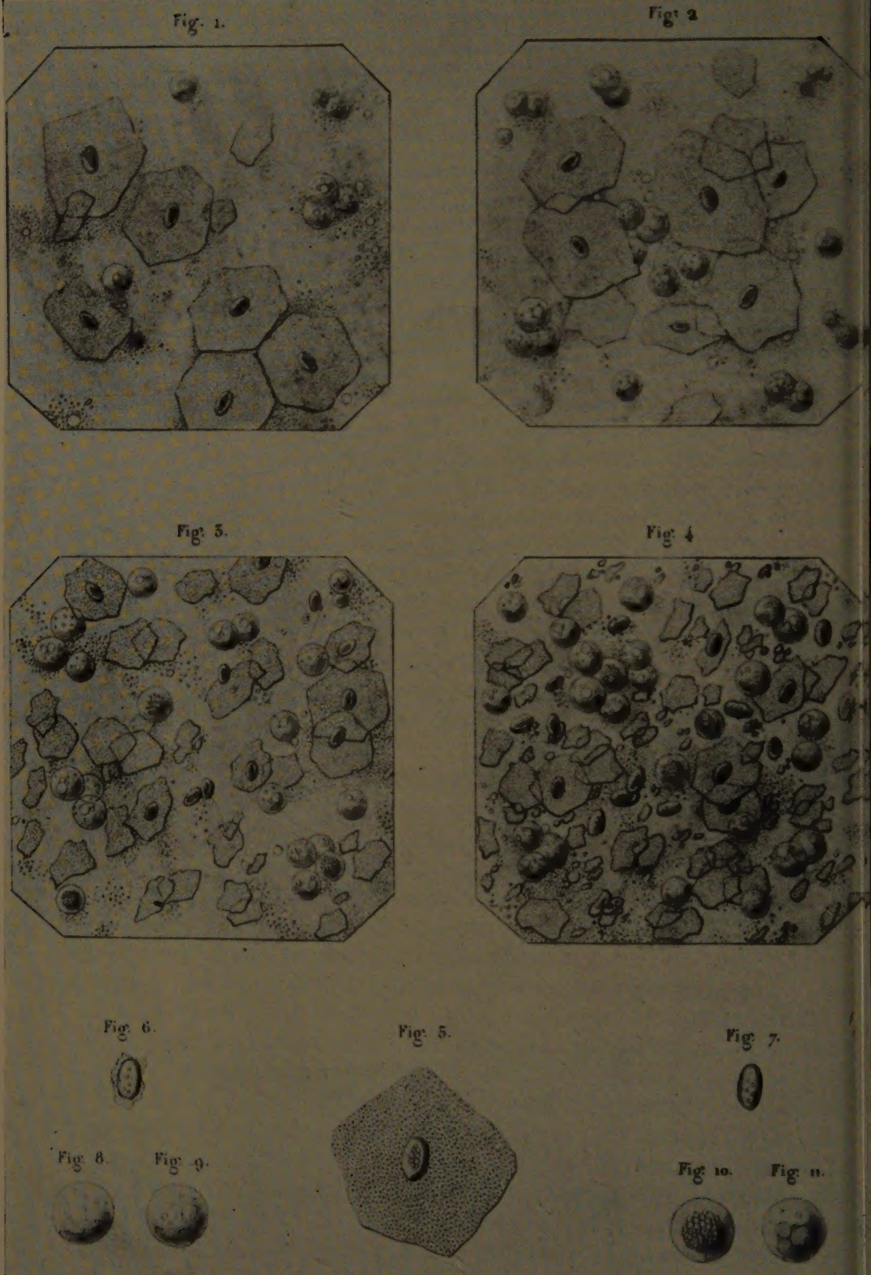
Menstruation de la Femme.



Pouchet del.

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Oudet

Intermenstruation de la Femme.

in 1838, published his studies on the microscopic aspects of tumors.[3] Müller was able to identify nuclei, granules, fibers, and crystals in the cells of tumors he studied. Isolated as well as small aggregates of cells are shown in his drawings. Moreover, even the nucleoli were seen in some of the cells as indicated in his figures.

Exfoliative cytology apparently was first applied by Pouchet in his studies on cells obtained from vaginal secretions as reported in 1847.[4] Of special interest are two plates, published in an atlas[5] (reproduced here as PLATES 1 and 2). Considerable cellular detail is revealed in these drawings, which were made from unstained specimens. This reference was recently drawn to our attention in a paper on the spontaneous biopsy by Adams.[6]

In the years that have elapsed since the publication of Müller's and Pouchet's studies, we have seen many remarkable advances. Practically everything we know about cells has been learned since then. For example, methods have been devised for studying living and fixed cells, at levels ranging from low-power microscopy to submicroscopic structures by electron microscopy. With such formidable background to draw upon, further research on living cells in which subcellular structures may be transplanted from one cell to another is now feasible. Even electronic devices have been developed, all of which give still more information on cells, both normal and neoplastic. The culmination in this important cavalcade of research from the point of view of instrumentation is the cytoanalyzer, so ably developed by Tolles.[7, 8]

The conference on which this monograph was based brought together experts in many medical and nonmedical disciplines. Those engaged in nonmedical research had numerous opportunities of visualizing and perhaps acquiring a sympathetic understanding of the problems faced by pathologists and clinicians. In a similar way, cytologists, pathologists, and clinicians have seen ways in which basic research may help in the solution of their particular problems and have thus set the pattern for active cooperation of all disciplines. Their teamwork is unquestionably required if the fight against cancer is to be a successful campaign.

The papers are grouped according to the session in which they were presented. It is believed that the papers in this monograph summarize and integrate the important knowledge of cancer cytology and cytochemistry, and thus provide an excellent background that we all need in order that our own research may be properly oriented. From many papers, new ideas and research in basic areas, as well as applications, will undoubtedly emerge. If this monograph can help in pointing out new pathways for productive research, then all efforts expended in organizing and holding of the conference will be amply repaid.

Acknowledgments

The conference on which this monograph was based, was developed over a period of nearly two years through informal consultation and correspondence

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PLATE 2. Reproduction of Plate XIII from Pouchet's atlas.[5] This plate shows the appearance of epithelial ells and "mucous globules" during periods of intermenstruation. Of interest is FIGURE 5, which shows an epihelial cell under high magnification to show the finely granular surface. FIGURE 6 is a cell fragment with a ucleus, while FIGURE 7 is essentially a free and modified nucleus. Original size of FIGURES 1 to 4 was 60 mm. cross. (Courtesy of the New York Academy of Medicine Library.)

with several individuals including: Doctors J. Ernest Ayre, Charles S. Camero Emerson Day, Ruth M. Graham, Raymond F. Kaiser, Paul Klemperer, J V. Meigs, George N. Papanicolaou, and James W. Reagan. The Conferen Chairman is deeply indebted to these individuals for their many helpful su gestions.

The Conference Chairman wishes to thank the Session Chairmen, and a participants for the care in preparing their papers, not only for presentati at the conference, but also for publication in this monograph. It would difficult indeed to find a greater spirit of cooperation than was evident amo the participants of the conference.

The conference was co-sponsored by the Damon Runyon Memorial Fu for Cancer Research, Inc., New York, N. Y. The Chairman, on behalf of t New York Academy of Sciences, wishes to acknowledge with appreciation t generous grant (DRG-301) made available by the Damon Runyon Memori Fund and also a grant from the Public Health Foundation for Cancer a Blood Pressure, Inc., New York, N. Y. for holding the conference. The grants to the Academy have assured the early publication of the proceedin of this important conference.

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THE HISTORY OF THE INTER-SOCIETY CYTOLOGY COUNCIL

By Joe V. Meigs
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In 1949, J. Ernest Ayre, Kenneth S. MacLean, John R. McDonald, and Harold L. Stewart decided that there should be some organization to stimulate interest in the new conception of cytology. They wished to make it possible for various institutions and hospitals throughout the country to use this new diagnostic tool. Doctor MacLean wrote me that he thought that Harold L. Stewart was the father of this particular idea for cytology. My introduction to the problem was in 1949, in London, where Doctor Ayre asked me if I would be a member of a steering committee to decide what to do about a cytology institute. He gave me a letter from Isador Rubin, urging that I accept a position on this steering committee. I accepted.

At a meeting in New York, Doctors Ayre and MacLean discussed the situation with me and, at the first meeting of the entire steering committee, I was made chairman. It was decided at that time to draw up a charter to be acted upon by the legislature of the state of New York. This was done, with excellent legal advice, and a charter was drawn up for the creation of the Cytology Institute. It was then decided that the membership should be enlarged and that certain selected people in the United States should be asked to join.

As chairman of the Cytology Institute, I found it impossible to interest a sufficient number of key people in the organization as it was constituted at that time. Further meetings were held and, eventually, it was decided to give up the idea of the Cytology Institute. Later, under the instigation of certain members of the steering committee of the Cytology Institute and members, as it was then constituted, another committee was constituted to reconsider plans. Arthur Purdy Stout was named chairman of this committee[1, 2] and, at a subsequent meeting in New York, plans were discussed. It was decided to appoint an investigative committee to consider what should best be done. This committee, called the Special Cytology Committee, was headed by F. Bayard Carter, of Duke University, Durham, N. C. The committee decided that an entirely new group should be invited to consider the growing interest in cytology.

From the first, it was not feasible to interest Doctor Papanicolaou in the Cytology Institute, and we felt that without him it would be impossible to proceed. At a meeting of the Special Cytology Committee, the new group was brought together at the Sloan-Kettering Institute in New York for consideration of the problem.

It was decided by this committee that we should form the Inter-Society Cytology Council (this name, if my memory serves me correctly, was first suggested by Frank W. Hartman of Detroit, Mich.), the new group participating in the foundation of the Inter-Society Cytology Council to be known as the Founders' Executive Committee. Joe V. Meigs was selected as chairman, and George N. Papanicolaou, Frank W. Hartman, F. Bayard Carter, Paul F Fletcher, Arthur Purdy Stout, Lewis C. Scheffey, Emerson Day, Charles S Cameron, and Raymond F. Kaiser as members of the Founders' Executiv Committee. Paul F. Fletcher, who was made secretary-treasurer of th Council, drew up a constitution for study by the Founders' Executive Committee. In September 1952, the constitution and by-laws were passed an became the Constitution of the Inter-Society Cytology Council.[3]

According to the committee, the aims of the Inter-Society Cytology Counc are that it should organize for the purpose of increasing interest in the cytologi method, so that the maximal potential usefulness of this method as a stud tool and as a clinical diagnostic technique may be achieved in as short a tim as possible. The Council is to encourage a high degree of scientific, academi and clinical interest and, at the same time, render maximum service, makin the cytologic method universally available.

The active participation of cytologists, pathologists, and clinicians in th organization and function of the Inter-Society Cytology Council is essentia No cytologist, pathologist, or clinician can utilize the method effectively without the other two. It requires the complete and unselfish help and cooperation of all three to assure the success of this new cytological organization The active membership of the Council is open, therefore, to clinicians, pathologists, and cytologists engaged or interested in an efficient utilization of th cytologic method. Its purpose is to make cytology generally available in th fight against cancer.

One of the Council's major aims in accomplishing this end is the subsidize training of capable cytologists. At the time the organization was founde there were only a handful of efficient exfoliative cytologists in the country The demand is great and the supply is inadequate. It is possible and probab that there are unqualified individuals who represent themselves as expert The general public cannot recognize them for what they are. The Inte Society Cytology Council hopes that training centers with adequate faciliti can be established, where instruction is free and a subsistence stipend is avai able during the period of study. We believe it is impossible to get this financi aid from existing agencies until minimum standards are established. The two related problems urgently require solution. Money is available to pr vide the training of physicians in cytology, but there has not been a gre demand for it. What is needed is money to support the laboratory as a trai ing station and technicians anxious to take such training.

Various other functions are outlined in the Society's constitution. Some these functions are as follows: (1) to encourage the establishment of addition cytologic facilities at local and regional levels, in medical schools, clinics, pathology laboratories, *etc.*, throughout the country; (2) to encourage the esta lishment of addition educational and training facilities for potential cytologist cytopathologists, and cytologic technologists; (3) to assist in formulating bas standards for early cancer detection, diagnosis, teaching, training, and r search, and to encourage the maintenance of such standards; (4) to advis foundations and governmental and voluntary or other agencies when the request counsel in the granting of funds for the establishment and operatio of the new laboratories that will conform to standard cytologic requirements; (5) to encourage programs of research, including the study of those problems that will be elucidated by the application of the cytologic method; (6) to assist in the preparation of an educational program in cytology for presentation to the public through the medium of the American Cancer Society and similar organizations; (7) to co-ordinate the activities of present cytologic laboratories in order to find a solution to the many difficult problems encountered in teaching and training of personnel; and (8) to recommend the establishment of a terminology for cytologic reporting.

Great care has been taken to insure the inclusion of all three groups, cytologists, pathologists, and clinicians. The president of the Council can be a member of any one of the three specialities. Besides the president, there are three vice-presidents, each one representing the clinical, cytologic, or pathologic specialites. Committees have been appointed to study standards and qualifications, to arrange for annual meetings, to decide what to do about the publication of papers read before the Council, and to decide who should and who should not be made members of the Council. Generally, it is felt that every one interested in cytology or engaged in some phase of cytologic activity should be invited to apply for membership, regardless of the extent of his actual knowledge and training in cytology.

In addition to medical personnel, *i.e.*, men who are doctors of medicine, it was felt that there should be a definite place in the Council for the so-called cytologic technologists or screeners, and that the latter should be included in the membership. The exact type of membership has not been decided as yet, and this is one of the questions that must be decided in the future. It is felt that this organization is fundamentally a medical society that should be run by doctors of medicine, but that there should be a category of membership including those who are not doctors of medicine but may be doctors of philosophy or science, also screeners, or those interested only in the technical point of view of detecting cells but without knowledge of other medical applications of the method.

When the Council's constitution was approved and the officers elected, Joe V. Meigs was made president; F. Bayard Carter, vice-president representing the clinicians; George N. Papanicolaou, vice-president representing the cytologists; Frank W. Hartman, vice-president representing the pathologists; Paul F. Fletcher was named secretary-treasurer; and Ruth M. Graham was made assistant-secretary to Doctor Fletcher.

In Philadelphia, at the first meeting,[4] the officers were re-elected, but at the meeting in Boston,[5] Arthur Hertig was elected president of the Council.

It has been felt rather definitely that there should be no Qualifications Board sponsored by the Council and that there should not be a Cytology Journal at the present time. All agreed, however, that the papers read at the meetings should be gathered together and published. This is being done as regards the papers presented at the Boston meeting in 1954.

It has been a definite aim of the Council to announce that the annual meeting is not the Council's only service. We hope that its greatest service will be the establishment of methods of training, of standardization of laboratories, and of making cytology and cytologic diagnosis available to the people of th country. It is disconcerting to think that this cytologic tool, which rende possible an early diagnosis of cancer in the female pelvis and cancer in oth locations, both female and male, is not available to every one in our countr It is the aim of the Council to insist upon satisfactory qualifications and t make it possible for more and more patients to avail themselves of this diagnostic technique.

Our scientific sessions have certain definite channels of presentation. Ne investigations are reported, and old methods that have been proved are presented statistically. It is our hope that there will be a succession of papers general interest, statistical as well as research and physiologic, thus assuring rounded view of the problems of cytology

Those present at the two meetings of the Council cannot but appreciate th superior type of paper presented. Those of us who attended the meeting i Philadelphia[4] realize that the standards set were high. Many of us reali that the meeting in Boston[5] was one of the finest meetings that one coul possibly attend. The interest was great, the meeting well attended, and th caliber of the papers superior. Clinicians, pathologists, and cytologists agre that this is a society that is well on the way towards being an important pa of the medical world.

The third meeting of the Inter-Society Cytology Council, under the pres dency of Arthur Hertig, will be held in Cleveland.[6] It is our hope that th meeting will again demonstrate the great interest and promise that the oth two have shown.

The aims of the Council are definite, and each particular facet is graduall being worked out by committees made up of those interested in each problem The Founders group of the Council is important. It represents those who interest in cytology has been great over the last decade. There is a lar group of members of our own country and, in addition, a group of foreign members who have been asked to join, who have the privileges of the Council, e cluding that of voting.

The foreign group is increasing in number, and its interest in the Coun is great. It was our pleasure, last year in Boston, to invite such men as Sta ley Way of Newcastle-on-Tyne, England, Alfred Glucksmann of Cambridg England, and Ollie Kjellgran of Sweden to present papers and to discuss problems. These men were important in the meeting, and their papers were hea with great interest.

The Inter-Society Cytology Council is on the way to success. It remai for its members to have a serious interest in the Council and to see that i standards are kept at a high level. From time to time, recommendations wi come to the members from the Executive Committee of the Council, advisi as to methods of increasing the importance of cytology, of increasing th number of technicians available in various parts of the country, of adding t the number of laboratories, and of maintaining high standards that will assu accuracy of diagnosis and safety to our patients.

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MALIGNANT PROPERTIES OF CANCER CELLS

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This is an elementary introduction to cancer cells, given as a kind of preface to a monograph by experts on the subject. What I have to say is really a brief summary of my book, *Cancer Cells* (1955). To distinguish between the important and the less important malignant properties of cancer cells and to present them in the space at my disposal is difficult. Fortunately, this whole monograph, *Cancer Cytology and Cytochemistry*, is a product of investigators who for years have focused their attention on cancer cells from many angles

By cancer cells, we understand the cells that characterize malignant tumors irrespective of the nature of their cells of origin. In other words, cancer cells include both carcinomas and sarcomas. By malignant (L. *malignus*, wicked) we mean tumors that grow at the expense of the body and, unless destroyed prove fatal. Tumor cells often invade neighboring tissues. There is much difference of opinion about them.

By some workers, ability to spread and produce secondary tumors in othe parts of the body is regarded as a criterion of malignancy, but tumors of the central nervous system considered malignant rarely metastasize in this fashion Such tumors cause death chiefly by the occupation of space needed by nerve cells.

Other workers identify the essential feature of malignancy as autonomou cellular multiplication uncontrolled by the usual regulatory mechanisms These cells, nevertheless, are subject to some control, because the rates o tumor growth sometimes vary between dormancy and rapid increase in size Having started on its antisocial career, the strain of cells continues to display malignant properties that have been ingrained in its constitution.

Assertions are made that, if our knowledge of cell division and resulting growth were only sufficient, we should quickly discover how to combat malignant growth. Consequently, the Committee of the National Research Counci that makes grants for cancer research and is financed by the American Cance Society is called the Growth Committee. Steiner (1954) says: "The attractive statement that the problem of cancer is primarily a problem in growth is both partly true and partly untrue, but it is incomplete and oversimplified."

It is safe to say that, owing to loss of specialized structures, or of failure to develop them, cancer cells resemble each other more closely than the norma cells from which they are derived.

This decrease in specialization is referred to as dedifferentiation. Befor we can evaluate dedifferentiation, however, it is necessary to have fairly clea ideas about what is understood by the term "differentiation."

In his classic monograph on cellular differentiation, Conklin (1924) define "differentiation" in the following words:

"All differentiation is transformation from a more general and homogeneou to a more special and heterogeneous condition. Dedifferentiation, on th other hand, is the reverse of this. Many differentiations are purely temporary, others are more permanent but, in general, protoplasm is not constant or static with respect to differentiation, but oscillates between different phases. These oscillations or cycles may be short as in physiological processes of assimilation and dissimilation, contraction and expansion, mitosis and intermitosis; or they may be long as in the life-cycles of individuals in which differentiation may be more or less permanent and irreversible."

A quarter of a century later, Weiss (1949) expressed his ideas of differentiation in terms of molecules. First, he proposed "a statistical concept of the cell which can best be characterized as ecology of an organized molecular population." The term ecology is derived from the Greek, *oikos*, household affairs, family, + *logia*, knowledge. It means the relationship of organisms to one another in one happy, well-organized family. According to Weiss, "Each cell and organized cell part (nucleus, chromosomes, *etc.*) consists of an array of molecular species whose densities, distribution, arrangement, and groupings are determined by their mutual dependencies and interactions, as well as by the physical conditions of the space they occupy. These species range from the elementary inorganic compounds to the most complex 'key' species characteristic of a given cell." Growth in these terms is defined as "the increase in the size of a given molecular population without essential change in character; differentiation as a progressive change in the composition of the molecular population including the appearance of new and the disappearance of old species." Weiss specifically stated, "Cellular differentiation must not be identified with the appearance of microscopically or otherwise discernible differentiation products (*e.g.*, pigment, myofibrils, secretion granules, *etc.*). Such products are merely criteria of a previous change in protoplasmic constitution (*i.e.*, composition of the molecular population) by virtue of which those products can be realized, provided certain accessory, nondeterminative factors are present (*e.g.*, oxygen for melanin, stretch for myofibrils, hormones for secretion, *etc.*). But even failing to materialize such overt terminal transformations, the various cell strains of a metazoan organism are differentiated among one another, that is, consist of molecular populations of different composition."

It seems to us that these "overt termination transformations" are too lightly dismissed. Without them, there would be no clues to the underlying differentiation that is said to consist of differences in composition of molecular populations. To set aside, as criteria of cellular differentiation, microscopically or otherwise discernible differentiation products such as secretion antecedents leaves us in a rather helpless position. Moreover, the degree of dedifferentiation can be measured, in practice, only by the disappearance of detectable differentiation products. This may depend either on a basic change in the molecular population, or on influences bearing on this population such as oxygen supply, mechanical factors of stress and strain, and hormones, or on both endogenous and exogenous factors. To work with discernible differentiations or their absence is not to dismiss without consideration the concept of Weiss of molecular populations.

For us, in our study of cancer cells, differentiation is the acquisition or possession of a structure or a function by a cell that it did not have previously. In other words, a cell that has differentiated has become different in some particular property or group of properties. But a senile cell, such as a corneal epidermal one, differs from the more vigorous spinous cells. In general, however, it has lost some features accompanying full cellular adulthood, so that we do not describe it as more differentiated. Senility and differentiation are not synonymous.

Consider, first, certain rather obvious properties of malignant cells, one by one, as compared with those of their normal prototypes.

(1) Malignant cells, taking them all together, show a smaller range of diversity of size and shape than normal cells. This is because they have lost, or retain to but a small degree, the special functions that are inseparable from special sizes and shapes. For example, they do not store within themselves large volumes of materials. Neither are they greatly extended in length for conduction, like nerve cells. But, in a given cancer, there is often greater diversity of size and shape of the malignant cells than is exhibited by the particular cells of origin.

(2) The cell membrane of malignant cells on microscopic study looks very much like that of their cells of origin. There is a chance, however, that structural differences exist that may be revealed by special techniques including electron microscopy. Some differences have been reported in the permeability of particular types of malignant cells as compared with their cells of origin. A difference has not been found, however, between all cells that are malignant and normal cells.

(3) If intranuclear viscosity can be measured by the extent of displacement of stainable nuclear contents under ultracentrifugal force, this viscosity is less in malignant cells of squamous cell cancers than it is in normal epiderma cells, but it has not been determined whether this low viscosity is a genera and distinctive property of malignant cells as a whole.

(4) Some mineral constituents determined by microincineration or chemica analysis, especially calcium and magnesium, are less in amount in malignant epidermal cells than in normal ones. They are, however, unusually abundant in normal epidermal cells to start with; that is, before the malignant transformation took place. Comparison of malignant cells with normal prototypes low in these constituents, as in hepatic cells, reveals little if any consisten difference.

(5) Mitochondria participate very actively in cellular physiology. Techniques for their study in living cells and in fixed preparations are excellent and they can be isolated from other cellular components and analyzed chemically. We should certainly expect to exist in them modifications that condition or accompany malignancy. Results are eagerly awaited.

(6) Because there is no simple technique for the microscopic demonstratio of the Golgi apparatus and because methods for its collection *en masse* by differential centrifugation for chemical analysis are only now being introduced no really significant results have been secured in comparing this structure a it exists in malignant and normal cells.

(7) Concerning the endoplasmic reticulum and growth granules, demonstra

ble by electron microscopy, no conclusions as to significant differences between malignant cells and their normal prototypes are warranted.

(8) Ergastoplasmic sacs, revealed also by the electron microscope, have limiting membranes of high electron density rich in ribonucleic acid. No data are available on any difference in these membranes between malignant and normal cells. Their discovery is very recent. But quantitative differences have been reported in the ribonucleic acid content.

(9) The marked proximodistal polarity of many normal epithelial cells is lost when they become malignant. This is sometimes evidenced by lack of uniformity in the position of the centrioles.

(10) The rongalite white and tetrazolium tinctorial reactions are not specific for malignant cells.

(11) Nuclear size and shape vary within wider limits in cancer cells than in the cells of the tissues from which they develop. They are less uniformly molded to discharge a specific function or functions than the cells of their tissues of origin. In fact, the tendency to uniformity occasioned by a common function inherited from their ancestral cells is often entirely lacking, since the said function has been completely lost. But a greater cause of the diversity in size and shape of the nuclei of cancer cells is the fact that many of them exhibit nuclear abnormalities and some of them are certainly dying. In general, the nucleocytoplasmic ratio is greater in cancer cells than in their nonmalignant forebears.

(12) The nuclear membrane of cancer cells is in structure not noticeably and consistently different from that of normal cells when studied by the methods of observation thus far employed.

(13) In respect to chemical composition, no substance has been found in the nuclei of cancer cells that is absent in those of their normal prototypes. Neither has any substance present in the nuclei of normal prototypes been proved to be absent in those of cancer cells. But the nuclei of cancer cells usually stain strongly with basic dyes, not more strongly, however, than the nuclei of certain normal cells. It is unusual to find cancer cells possessed of very pale staining nuclei. As a group, they are rich in ribonucleoprotein.

(14) The nucleoli of the cells of some cancers are conspicuous by their large size. Some nonmalignant cells, particularly hepatic cells, however, have equally large nucleoli, so that this feature is not distinctive.

(15) False nucleoli (chromocenters) in cancer cells, though they are often outstanding in appearance, apparently show no significant differences from those in normal cells.

(16) Chromonemic threads, in a sense, are the backbones of the chromosomes. The chromosomes in cancer cells are much more diversified than in the corresponding normal cells, but a distinctive deviation from normal in their chromonemic threads has not been discovered.

(17) The nuclear sap of the malignant cells of squamous cell carcinomas may be more fluid than that of normal epidermal cells, because the nuclear contents are more displaced by ultracentrifugal force; but the presence of this difference in other cancers has not been investigated.

(18) Nuclear division is frequently more irregular in cancer cells than in

normal ones, but there is no feature invariably present in one and absent in the other.

(19) Mitotic times of both metaphase and anaphase, as measured by Lewis (1951) in cultures of malignant fibroblasts are longer than those of normal fibroblasts. How widespread this difference is in other cancers we do not know.

(20) Rhythms in mitotic frequency have not been clearly described in cancer cells, though they are exhibited by several types of normal cells.

(21) Mitotic poisons that are not carcinogens, acting on normal cells, are apparently capable of producing all of the mitotic abnormalities observed in cancer cells. Since cancer is not produced, but the abnormalities are, the question is raised whether the abnormalities themselves bring about malignant transformations. These poisons may be of use in cancer therapy; for cells, at some stage in the process of division, seem to be especially susceptible to them and, in rapidly growing cancers, there are many multiplying cells.

In summary, the following more or less distinctive properties are usually exhibited by cancer cells: (1) relatively high water content; (2) motility and invasiveness; (3) multiplication unregulated by the usual controls; (4) short individual lives during which cytoplasmic storage is at a minimum; (5) active protein synthesis, indicated by intense ribonuclease activity and large nucleoli, monopolizing nutritive materials needed by all other cells.

Cancer cells appear in varieties probably much more numerous than the kinds of normal cells from which they develop. Thus a great many sarcomatous tumors showing distinct individualities have resulted from the malignant transformation of fibroblasts under the influence of chemical and physical carcinogens. Perhaps, each and every cancer is an individual, and this, together with differences in the stroma, may explain the differences in behavior between cancers. Differences more subtle than those manifested by deviations in structure or in behavior are expected.

The concept of stemline cells from which tumors are propagated, developed by Makino and his co-workers, is interesting (Makino and Kanô, 1955). It is based on cytological and statistical studies of ascites tumors in rats yielding evidence of the continued existence of tumor-cell populations, "high frequency of occurrence, definite chromosome patterns persistent through serial transfers and regular mitotic behavior." The characteristics of the stemline cells remain unchanged despite heteroplastic transplantation and treatment with chemicals.

The investigators mentioned have isolated two single-cell clones. The word "clone" is employed by geneticists to indicate "a group of individuals of like genetic constitution obtained by asexual reproduction from a single original individual" (Blakiston). It is derived from the Greek *klōn*, a twig. The single cell inoculation was made after microisolation of a Hirosaki sarcoma cell of an ascites tumor of mixed stem lines in rats. This was successful in 4 out of 51 trials. Each single-cell clone was characterized by a chromosome pattern structurally distinguishable from the others; and a tumor established with a two-cell inoculation of 2 V- and 3 V-types of cells developed respectively two different neoplastic populations of definite frequencies in each population.

The point is that, in humans, as well as in rats, some, perhaps many, cancers are made up of mixed stemlines. The properties of the cellular components of the mixture and their relative frequencies are probably of great consequence in molding the behavior of the malignant tumor as a whole.

It is not possible to single out any particular property of cancer cells and explain that this is why they do so much damage. Their behavior depends upon their genetic constitution and their tissue fluid environments. Spread by metastasis is often a manifestation of the malignancy of cells of primary cancers, though it is generally suppressed by transplantation. Behavior in other ways is modifiable to some extent.

All cells in a given cancer do not behave in the same way. Some die without offspring, and a few lose their malignancy through differentiation, as in the formation of epithelial pearls. Perhaps the giant cells formed from truly malignant cells are not themselves malignant. Efforts to combat malignancy by promoting differentiation have not yielded promising results.

We must direct our attention to the normal cancer cells; that is, to the majority that give character to the particular cancer. The cancer cells are abnormal in respect to the nonmalignant cells of the body in which they make their appearance. They are probably mutants, and what is normal or usual among the descendents of the particular mutants is the question. We want to investigate the properties of these cells in the full flush of their individual malignancy, setting aside those which, for some reason or other, are degenerating and dying. Tumor cells, according to Ewing (1940), "suffer the same degenerative changes as normal tissue," whereas Fischer (1937) stated that while "normal cells remain for a long time among living cells in a rather inert state, the body of the dead cancer cell is readily hydrolysed and soon disappears." The point is that one should be careful not to compare a degenerating malignant cell with its normal prototype—a possible mistake when very high magnifications are employed for cellular detail and comparatively few cells are studied.

Malignant cells can live in all tissue fluid environments. They are very adaptable It is not surprising, therefore, that they become resistant to chemotherapeutic agents which at first injure them. The same holds in their change from radiosensitivity to radioresistance. Similar acquired resistance may face attempts to kill off cancer cells by viruses. These particulate agents enter the cancer cells and seem to destroy them by their rapid multiplication and utilization of nutritive materials needed for survival by the cancer cells. Such viruses starve the cancer cells as the cancer cells starve the body. Time may be insufficient for adaptation.

The reason why the body is incapable of defending itself against malignant cells that prey upon other cells in the community almost as parasites is that the malignant cells, though they have gone wrong, so to speak, are nevertheless members of the community and are accepted by it. Immunological defenses are not called into action as would be the case if the malignant cells were foreign invaders from some other individual or race. Indeed, not only are the cells of one primary cancer received without resistance, but a second primary cancer is definitely more likely to develop in a person having one primary cancer than in a person free from cancer (Warren, 1953). The host parasite idea of the body-cancer relationship gives a false setting to the problem.

I have mentioned only a few of the more obvious malignant properties of cancer cells. Details will be presented elsewhere in this monograph by others who lead in their fields of investigation. Evidently malignant cells are identifiable not by the estimation of one, but of several criteria. It is unlikely that any individual premalignant or precancerous cells can be surely recognized under the microscope, even though many properties are examined. Yet it is a matter of great importance to be able to detect precancerous conditions, among which is usually listed uterine carcinoma *in situ*

Much of the discussion at this conference deals with the so-called "precancer cell" of the cervix and body of the uterus. Precancerous lesions can be recognized in many parts of the body, and the uterus is probably no exception. They are defined as altered areas of tissue in which cancer develops more frequently than in normal areas of the same tissue. It is to be noted that cancer does not always appear in any known type of precancerous lesion even when the possessors of the lesion live to extreme old age. Consequently, not all precancerous lesions are, in fact, precancerous; that is, they are not followed by cancer.

A particular precancerous lesion in which a tiny primary cancer has developed is not to be considered as having been packed with "precancer cells." I am content with the evidence that the malignant transformation is a mutation, and that a particular mutation takes place only in very few of thousands of susceptible cells subjected to the action of a mutagen, be this X rays or something else. To designate all atypical looking cells in a precancerous lesion "precancer cells" is, in my opinion, going too far. The chances are that the vast majority of them, and/or their descendants, if they had not been removed and killed, would not have given rise to cancers. At present, we cannot tell which of them, if any, is really in the line of cancer production. At most, they merely resemble cells whose presence is associated with a higher incidence of cancer than is found in their absence.

Our group in St. Louis has done a lot of work attempting to trace sequences in experimental epidermal carcinogenesis. By many techniques, we have learned how to recognize precancerous epidermal hyperplasia chemically, but to point to any individual epidermal cells microscopically in such lesions and to identify them as precancer cells is beyond us. Since chemical determinations especially of enzyme activities, using very small pieces of tissue, may yield significant data, the chemical study of cervical biopsies may supplement usefully the microscopic examinations of smears, at least in special, limited investigations. This, however, will not replace the examination of smears that is so essential on a large scale to detect cancers while they still can be cured.

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EARLY UTERINE CANCER DETECTION BY CYTOLOGIC SCREENING*

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It is my assignment and privilege to discuss the objectives and problems of mass or population screening using the exfoliative-cytology genital-smear technique for detecting early uterine cancer, and to present a preliminary report of progress in a project designed to evaluate the effectiveness of such a screening survey program in Memphis and in Shelby County, Tenn. The ultimate goal of the successful application of this approach to uterine cancer control is clearly aimed at decreasing the high incidence of clinical symptomatic cancer by early detection of incipient curable cancer and precancerous or preinvasive lesions.

The project to be reviewed in this report is a two-pronged attack in the study of uterine cancer control: first, a project which has as its immediate goal an evaluation of the feasibility and reliability of the vaginal smear as a screening test; second, an investigation that will provide valuable information regarding age specific incidence and prevalence rates, and more accurate knowledge from general population data as a contribution to the problem of the relationship between intraepithelial and invasive carcinoma of the cervix.[7, 9, 11, 19]

The dictum "early cancer is curable cancer" establishes detection of preclinical or presymptomatic states of cancer as an essential feature of a good cancer screening method. Cytologic studies have contributed to focusing attention on the problem of intraepithelial carcinoma of the cervix and incipient preclinical cancer.[2, 3, 9, 12, 15, 17] Available evidence indicates that it is in finding "*in situ*" or intraepithelial carcinoma, whether it be a precancerous lesion or a preinvasive cancer, that lies the solution of the early detection of uterine cancer. As indicated above, the information is not yet available to settle many of the questions related to intraepithelial carcinoma,[7, 9] and I do believe that data to contribute to solution of these questions will be gained from successful continuation of this project. The current concept of the status of this much debated lesion is reviewed by several other contributors to this monograph. Let me say, however, that in regard to population screening for uterine cancer, it is essential that the thesis that "intraepithelial carcinoma is a significant lesion in relation to invasive uterine cancer" be correct if this approach to cancer control is to be acceptable and economically feasible.

This monograph on cytology ably tells the story of the development of the exfoliative cytology method described by Papanicolaou and Traut.[16] For genital cancer, this technique has been accepted and established as a useful, practical laboratory aid with a demonstrated potential for a high level of accuracy, at least in established cancer.[1, 2, 4, 10, 12, 15, 17] As a pathologic method for sampling, the smear test gives a diffuse and representative cellular speci-

* This investigation was supported in part by research grants from The National Cancer Institute of The National Institutes of Health, United States Public Health Service, Bethesda, Md.

men of the genital tract. From the patient's viewpoint, it satisfies important criteria as a screening or cancer test, for the test can be performed easily and quickly, and is safe. In a screening project or in a cancer test, we are interested primarily in a detection tool to select suspicious cases. We are not interested primarily in the accuracy of the vaginal smear as a diagnostic technique. It is my belief that this technique, applied to the reproductive tract, especially in screening, has its greatest potential usefulness in pointing "the finger of suspicion at cases showing cytologic abnormalities that deserve further study."

In addition to applying the criteria for evaluation of a cancer test, the feasibility and reliability of the mass-screening program are two of the basic aspects to be considered. It is clear that a significant percentage of the designated population of women must be reached, that facilities and personnel must be potentially available, and that application of this approach should be economically feasible for the individual or the community in relation to the total cost of detecting and treating uterine cancer.

Thus, in effect, mass screening for uterine cancer detection must point, efficiently, accurately, rapidly, easily, and economically at a significantly high percentage of unsuspected asymptomatic, incipient cancers, as well as at intraepithelial carcinoma. With the assumption that our present concept of carcinogenesis of cervical cancer is correct, successful screening by this method should potentiate, by removal or treatment of the "*in situ*" and incipient group, a change from the picture of a predominantly symptomatic noncurable disease to one where curable intraepithelial lesions or incipient uterine cancer predominate.[5, 6, 9]

Experimental Plan and Procedure

Can the vaginal cytology test be utilized in a screening program involving an unselected population of 200,000 women? Can a survey be carried out efficiently in predominantly asymptomatic or well women to detect uterine cancer? Can case-finding on a mass scale to accomplish effective detection of early disease result in presymptomatic diagnosis and subsequent treatment in a majority of women destined to have this disease?[9] To answer these questions was the immediate objective of the investigation initiated at the University of Tennessee in Memphis on July 22, 1952. The investigation has been directed by the staff of the Institute of Pathology at the University of Tennessee. I should emphasize that this has been a cooperative venture carried out with the joint participation of the staff of the Department of Obstetrics and Gynecology of this university, that of the Memphis and Shelby County Health Department, and of the National Cancer Institute. The National Cancer Institute has supported the project's cytology laboratory and has given financial aid in grants. The program has been sponsored by the Memphis and Shelby County Medical Society. Such co-operation by practicing physicians in the community is obviously essential.

The plan has been to examine by a single satisfactory vaginal cytologic examination as many women, 20 years of age and over, as were available in the Memphis and Shelby County area. Each suspicious and positive smear was to be followed by a cervical biopsy or other pathological tissue study as indicated. Thus, the initial aim of this study has been to reach as many women as possible in a designated area in order to test the vaginal smear as a screening test in a large unselected group of women. At the outset of the survey, the test population included approximately 163,000 women 20 years of age and over. This total is now estimated at close to 200,000.

In the second phase of this experiment, it has been our objective to reexamine these women by vaginal smear annually three times. As previously mentioned, I it is my belief that this annual retesting can contribute significantly to our knowledge of the pathogenesis of uterine cancer. Successful rescreening of this population of women can give information not now available regarding the true incidence of both intraepithelial carcinoma of the cervix and invasive uterine cancer.

At the present time approximately one half of the tests are made on private patients. Reports on these tests are obtained from the more than 250 actively participating Memphis physicians. Fifty per cent of the tests are made on women visiting clinics established for the purpose of taking vaginal smears established in health centers, in 51 city and county school clinics, in the City of Memphis hospitals, and on women visiting annual temporary clinics set up in industries throughout the city and county. Clinics arranged for a single day or several days, depending upon the number of women, were set up in industries where 50 or more women were employed. Sixty Memphis industries have participated in the project to date. The enthusiastic cooperation of these companies has been a significant factor in making this test available to a large group of Memphis women. I have referred to these details regarding the source of tests, for this project should provide data from a representative unselected population including all economic and racial groups, as well as from a high percentage of asymptomatic women. To accomplish this end, one of the major tasks has been to inform women of the opportunity available, and to stimulate them to go to doctors or to cytology clinics for the vaginal smear examination. That 95,000 of the test population has been screened within two and one-half years and that 27,000 women have been examined in the second annual rescreening indicates the progress made to date.

The organization of the cytology laboratory established for this program has been previously described.[8, 9] It proved pertinent, in planning for this screening laboratory, to refer to our experience, according to which the average screening capacity of one technician is 25 to 30 smear tests per day. The screening technician in our laboratory has the responsibility of determining whether the slide is negative, unsatisfactory, or shows abnormal or suspicious cells. It seems to be clear, and it is important, that such utilization of qualified screening technicians is essential for any widespread or adequate use of the cytologic method of screening. We have found that facilities must be maintained continually for the recruitment and training of technical personnel to maintain a full technical staff for a large screening program. The pathologist is responsible for final interpretation of questionable, suspicious, and positive cases, for reporting to the physician, and for consultation in correlation and follow-up studies in diagnosis.

The vaginal aspiration technique was chosen because of the ease and simplicity of obtaining specimens and its applicability to mass screening.[10] Technicians can be trained to obtain the vaginal specimen. In our laboratory, we classify the "essentially normal" smears as type 1. In type 2 smears, meaning "abnormal, possibly suspicious cells present," it is our procedure to request repeat vaginal smears, as well as cervical aspiration and cervical scraper specimens. In all those classified as type 3 and above, we "advise biopsy." No specific diagnosis is made to the physician, but only a report that the smear is negative or unsatisfactory, or that follow-up studies are indicated ("repeat smears," "advise cervical biopsy or uterine curettage"). The latter method of reporting to the doctor emphasizes our philosophy that in this project we are using the cytologic technique as a screening method and not as a diagnostic procedure. I emphasize the fact that, while exfoliative cytology has proved to have a high degree of accuracy in detecting invasive cervical cancer,[2, 10, 16, 17] it seems clear that its greatest potentiality lies in identifying "borderline" questionable lesions of the cervix and varying stages of the intraepithelial lesion that are not so accurately classified by the cytologic technique. It is to be expected that increasing the sensitivity of the technique by obtaining biopsies in all cases considered cytologic type 3 and above will not only increase the "unsuspected" incidence but will also increase the number in which the biopsy will not reveal cancer.

Our classification of smears in which biopsies are recommended is: type 3, abnormal cells, moderately suspicious; type 3a, suspicious of intraepithelial type cells; type 3b, indicating intraepithelial type cells; type 3c, very abnormal cells, suspicious of invasive carcinoma type; type 4 and 5, thought to be positive for malignancy.[2, 16] In evaluating for correlation of cytologic and pathological findings in our laboratory, we consider type 3 and 3a "suspicious"; type 3b and above, "positive."

Preliminary Summary of Results and Progress

The women examined by at least one vaginal examination total 95,000. Table 1 shows the results of the first screening and the pathological findings of biopsied cases in the first 80,000 women. In 1,452, a rate of 18 per 1,000, a biopsy was recommended; in 1,200, or 15 per 1,000, the biopsy was completed. This represents an 83 per cent rate of successful follow-up study. The rate of four intraepithelial carcinomas per 1,000 is an index of the prevalence of "*in situ*" disease for this population in this period. The number of invasive carcinomas that are listed represents only cases that were examined by smears in this study, correctly interpreted, and subsequently confirmed. The ratio of intraepithelial carcinomas to invasive cancer refers only to the tested group, and is not a significant index for this population. A significant number of obvious clinical cancers are not examined cytologically prior to biopsy, especially in the private patient group. The ratio will be evaluated at a later date, after a second morbidity survey for uterine cancer is completed for this population area similar to that conducted prior to the beginning of the project in 1951 and 1952.[5] Similarly, the tabulated data are not corrected for the 10 per cent of women included who are not residents of Shelby County. This 10 per cent nonresident group contributed 90, or almost one third, of the

TABLE 1

FOLLOW-UP RESULTS AND PATHOLOGICAL FINDINGS IN WOMEN WITH "BIOPSY RECOMMENDED" AS RESULT OF FIRST SCREENING OF FIRST 80,000 WOMEN EXAMINED BY VAGINAL SMEAR TECHNIQUE*

	No.	Per cent of total
(1) Number of women, "biopsy recommended"	1452	1.8
(2) Biopsies completed	1200	1.5
(3) Confirmed cancer and intraepithelial carcinoma of cervix	627	0.78
a. I. E. carcinoma of cervix	321	0.40
b. Invasive cancer, uterus	288	0.36
c. Other genital cancers	18	0.02
(4) Atypical metaplasia, cervix (dysplasia, "borderline," possible I.E. carcinoma)	144	0.18
(5) Inadequate biopsy	39	0.05
(6) No. I.E. carcinoma or cancer	390	0.49

* *Progress Report.* July 21, 1952 to April 22, 1955.

cancers shown, as confirmed in the 80,000. Such nonresident examinees are sent to us through the participating private physicians, and they show that a specialist's consultative practice, as well as patients of a hospital and clinic, will be highly selective. By contrast, the ratio of intraepithelial carcinoma in the resident and nonresident women was essentially the same as the rate for the total group. Approximately one third of women examined were Negro, and two thirds were white. A total of 145, or 45 per cent, of the cases of intraepithelial carcinoma were in Negro women. The latter data are of extreme interest, but must be evaluated further after coding analysis of the records has been completed.

Pathologic examination (TABLE 1, No. 4) of tissue biopsies revealed two women in 1,000 with atypical metaplastic epithelium of the endocervix. This group is considered "suspicious of intraepithelial carcinoma." I believe the detection of the latter lesions are particularly important. Identification for follow-up and subsequent studies of such "borderline" tissue changes can result in further elucidation of the earliest stage of cervical carcinogenesis.

TABLE 2 includes a summary of the known false negative cytologic reports in the first 80,000 women examined with relation to the first screening. This data is obtained by checking the records of cases of cervical cancer in Memphis hospitals and from doctors against the records of the cytologic clinic. This figure can be considered as a preliminary index only. We cannot now accurately say how many cases were missed in the 982 women in each 1,000 that were called negative. The false-negative value will be reviewed after annual rescreening. The screening examinations repeated annually will furnish the data to determine the number of cases that were either missed or developed during the intervening time period.

It may be stated that in a recheck of the screened smears from the false-negative group, at least 50 per cent of the smears had no abnormal cells and that 50 per cent contained abnormal cells that were classifiable as type 2.

Group 2a (TABLE 2), including type 3 and 3a smears, numbering 312, reflects our view that it is in this "borderline" suspicious group that a significant

TABLE 2

KNOWN FALSE NEGATIVE REPORTS IN FIRST SCREENING OF 80,000 WOMEN AND SUMMARY OF CYTOLOGIC CLASSIFICATION OF CASES WITH NO INTRAEPITHELIAL OR INVASIVE CANCER DEMONSTRATED IN CURRENT FOLLOW-UP TISSUE EXAMINATION

(1) *Known false negatives*	31
(a) Intraepithelial carcinoma of cervix	9
(b) Invasive uterine cancer, total	22
Squamous cell carcinoma, cervix	10
Adenocarcinoma of uterus	7
Other malignancies	5
(2) *"Biopsy advised," no cancer found*	
(a) Suspicious cytologic classification, type 3, 3a	312
(b) Positive cytologic classification, total	78
Type 3b, 3c	55
Type 4, 5	23

number of the early intraepithelial carcinomas and incipient cancers will be found. I again emphasize that the vaginal smear method in population screening is not used as a diagnostic test for cancer but as a screening method to select cases for further study by biopsy. We must also recognize that, in any mass survey, the problems increase proportionately, both as far as the doctor and the patient are concerned, as the number of repeat cytologic examinations are increased prior to making a decision to "advise biopsy." In the unconfirmed cytologically positive group (2b, TABLE 2), we include the smears interpreted as intraepithelial type, those suspicious of invasion, as well as those interpreted as "thought to be cancer." You will note that of 78, 23 were in the 4 or 5 cytologic category. One author has indicated that the false positives are more serious than the false-negative error.[17] With this view I disagree. It has been our experience that it is the false-negative interpretation that is upsetting to both patients and doctor. So long as the number of unsuspected early uterine cancers detected by the screening method is significant and the number of cases classified in the laboratory as false-suspicious is reasonable in relation to the number of biopsy cases, the criteria for a satisfactory screening method can be satisfied.[6]

FIGURE 1 indicates the number and percentage of unsuspected cases of disease that resulted from the vaginal-smear test in the first screening of 70,000 women. TABLE 3 shows similar data summarized for 80,000 women. The measure of whether a case is unsuspected is determined by asking the physician, "Would this patient have had a biopsy or been diagnosed at this time if the cytologic test had not been taken and biopsy recommended?" Thus "unsuspected" means that detection and diagnosis resulted from the exfoliative cytology report, "advise biopsy." These data show the most significant finding to date, that 60 per cent (374 of 627) cases of all women examined having intraepithelial carcinoma or uterine cancer detected by vaginal cytology were "unsuspected." These data indicate that a significant percentage of unsuspected cases may be detected by the method and under the procedure used. As might be expected, the intraepithelial group predominates in those women with unsuspected disease.

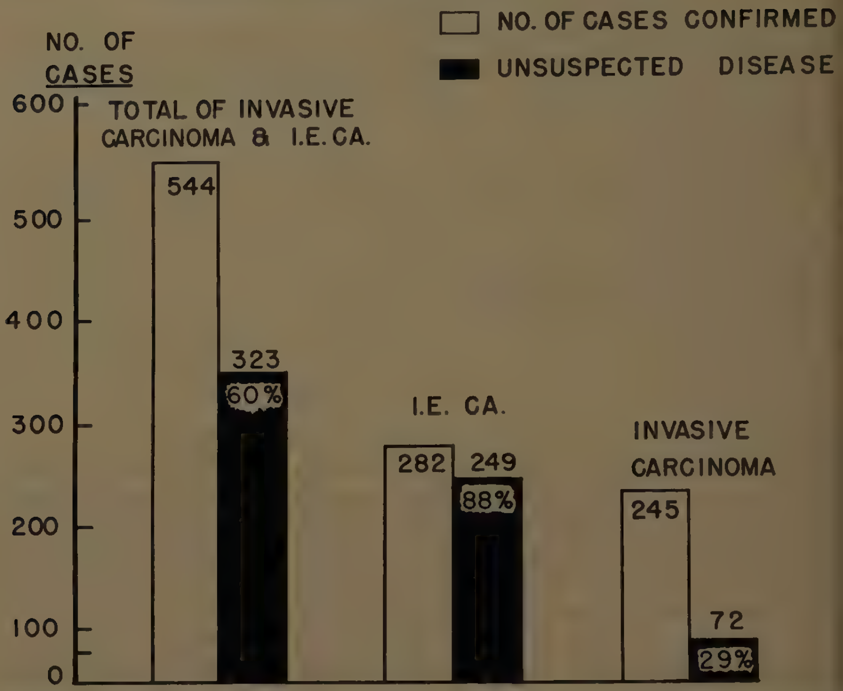

Figure 1. Women with unsuspected disease detected by survey examination compared to total number confirmed in first 70,000 women examined.

Table 3

Number of Unsuspected Cases of Intraepithelial Carcinoma and Uterine Cancer Detected by First Screening of 80,000 Women Examined

	Total No.	Unsuspected
Women biopsied	1200	
Uterine cancer and I.E. carcinoma of cervix, total	627	374 (60%)
Invasive cancer of uterus	288	87 (30%)
I.E. carcinoma of cervix	321	287 (89%)

Figure 2 shows the age at which intraepithelial carcinoma or cancers of the cervix were detected and labeled. This figure clearly emphasizes that any mass or population screening, to reach the important group with intraepithelial carcinoma, must include women under the age of 30 and, preferably, at the age of 20. You will note that the peak for intraepithelial carcinoma is between the ages of 30 and 35. The curves cross approximately at the age of 45, with the peak incidence of invasive carcinoma at the ages of 50 to 55. These data are plotted according to age and the number of cases in a 5-year period

Progress in Recalling Women for Annual Rescreening

Tables 4 and 5 reflect the problems and progress of the rescreening program Our original plan was to wait at least a year after examination and to stimulate

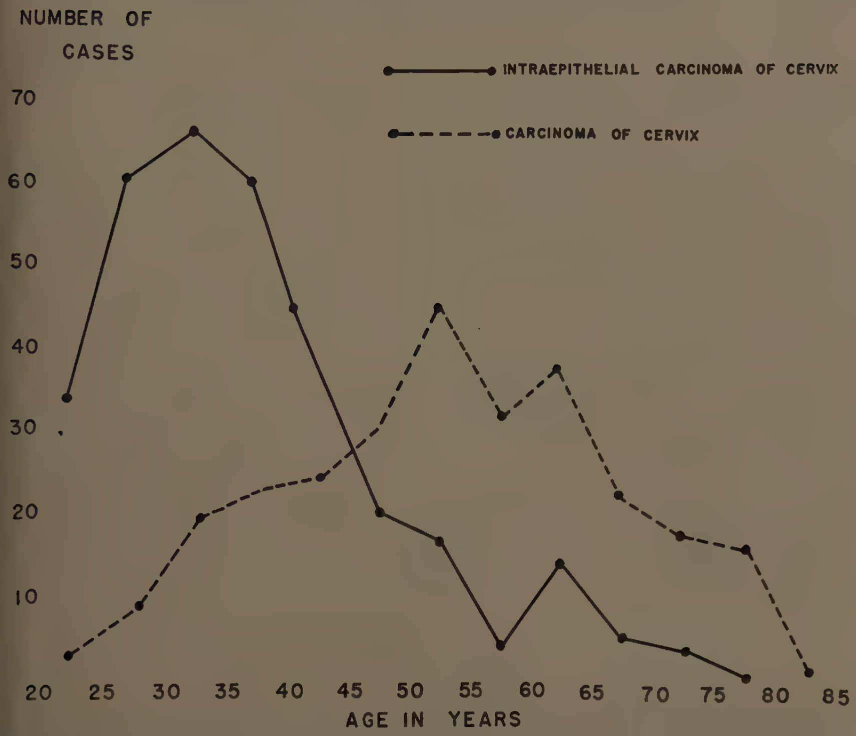

Figure 2. Age distribution of intraepithelial carcinoma and invasive squamous cell carcinoma of the cervix at time of detection in first 70,000 women examined.

Table 4

Recall Results of Annual Repeat Screening of 7 Illustrative Industrial Clinics. Per Cent of Women Accepting Opportunity to Have Annual Repeat Vaginal Cytology Examination

Location	Women tested		Per cent of repeat tests in 1954 clinic	Women tested 1955	Per cent of repeat tests in 1955 clinic
	1953	1954			
American Snuff Co.	78	74	64%	81	82%
Plough, Inc.	139	118	49%	122	64%
American Finishing Co	68	52	36%	52	69%
G.E. Lamp Works	126	153	61%	206	72%
Firestone Tire and Rubber	302	222	40%	136	44%
Kennedy Hospital (staff and employees)	214	251	43%	255	60%
Memphis General Depot	184	144	45%	165	61%

return of women to physicians or to clinics by personal letters from physicians, followed in two months by a reminder card and, in another month, by a home visit. It has become apparent, as in the group shown in TABLE 5, representing 3 groups of the first 32,000 women examined on the first screening, that to stimulate, remind, or educate women to return for annual reexamination is a major problem. In this effort, a letter, a reminder card, a

TABLE 5

PROGRESS IN RECALL OF WOMEN FOR ANNUAL RESCREENING[*]

No. of women	Group I 7,200 *Per cent*	Group II 10,667 *Per cent*	Group III 14,853 *Per cent*	Total 32,720 *Per cent*
1. Not located or deceased	13%	8%	3%	7%
2. No. of repeat tests prior to recall procedures	12%	26%	31%	25%
3. Response to letters, cards and home visits	30%	20%	15%	20%
4. Total No. repeat tests	42%	46%	46%	45%

[*] To April 22, 1955.

telephone call and, if the patient is not reached by these methods, a home visit, has resulted in rescreening approximately 45 per cent of the first 32,000 women. TABLE 4 is illustrative of the recall results in women having the opportunity to obtain repeat annual tests in industries. In 45 per cent of the first 30,000 women examined a second time, 0.19 per cent, or less than 2 in 1,000, had intraepithelial carcinoma or uterine cancer that was either missed the first time or developed in the interim. The *in situ* lesions were almost four times as numerous as the invasive cancers.

Discussion

Evaluation in this study of the feasibility of vaginal cytology alone as a means of case finding in a mass survey has been successful.[9] A total of 95,000 women, 50 to 55 per cent of the available female population, have accepted the opportunity and have been examined at least once during the past two and one-half years. Of this total, 27,000 women have now had a second annual test.

The cooperative participation by physicians in this community has been an essential factor in achieving these results. Equally important was the cancer-education program that had to be carried out in order to reach as many women as possible in the designated survey area and to inform them regarding the objectives of the project and the value of the test to the individual. We have likewise considered it just as essential to give to each woman having the test a memorandum indicating: that the examination is only for cancer of the uterus; that, according to present knowledge, the test should be repeated annually; and, last, that if any symptoms or signs of cancer develop, a physician should be consulted immediately. Regular announcements and descriptions of the project have been carried frequently in local newspapers, on the radio, and television. One member of the staff is a person who has had experience in public relations in the health field and it has been the responsibility of this individual to utilize judiciously the available information ch nels and to seek all opportunities to discuss and present the objectives a value of the project before women's organizations and before parent-teacl and church groups.[8] TABLE 4 clearly shows that, at least in the early part of this program, the great majority of women did not voluntarily return for a second annual examination. Thus, it appears that even after participating n one screening, these women were not sufficiently conscious of uterine cancer s a personal problem to return voluntarily. Suffice it to say that properly lanned stimulation and educational measures have been necessary to attain he annual rescreening of 45 per cent or more of the women originally tested.

Can the accuracy of this test for screening be maintained, so that but a ew cases of cancer will be missed? In any mass screening program, it is ssential that the test be so efficient that the number of misleading reports or nterpretations will be low. False security must not result from too many alse-negative tests. I have previously discussed the low known false-negaive error (TABLE 2), and the fact that rescreening will give additional data for nore accurate evaluation of this index. If a detection case-finding technique ollowed by tissue study for diagnosis results in a low false-negative rate and n the identification of a high percentage of cases of unsuspected early, localized ancer, then, in the "biopsy advised" cases, the false-suspicious and false-posiive error is required to be only within reasonable limits.

The philosophy of this project has been to evaluate and use the vaginal mear as a screen—as a means of choosing women in whom there is a reasonble probability of significant disease. To do this, the test spectrum must be road enough to include a high percentage of early or localized disease. Dunn nd Greenhouse[6] have set up criteria to follow in evaluating any cancer-screenng program. These authors show that, to be of value, a test must have no nore than 10 per cent false-negatives in early cancer, and that there be, at nost, 5 per cent false-positives. As previously indicated, the data indicated n TABLE 2 will be restudied after rescreening studies. It is apparent, however, hat the available data, including 0.04 per cent false-negatives and 0.5 per ent unconfirmed suspicious and positive cytologic tests, appear to be very atisfactory. These data, moreover, appear to constitute an excellent test as neasured by their criteria. Cuyler[2] pointed out that the derivation of the alse-positive error is not simple, and that it illustrated several means of deermining an index for incidence of error.

Is this survey productive of cancer case detection at an early stage in a ignificantly high percentage? The finding of 0.47 per cent of unsuspected ases of "*in situ*" cervical carcinoma and cancer, or almost 5 per 1,000 of 0,000 women examined, answers this question in part. FIGURE 1 and TABLE show that of the 627 proved cases, a high ratio of unsuspected disease (77 er cent) was of the noninvasive or intraepithelial carcinoma group, as one night expect. Unsuspected cases, however, have not been limited to the *in situ*" variety. Thirty per cent of the 288 cases of invasive carcinoma .36 per cent of the total number of women examined) were detected by neans of the vaginal cytology test, according to their physicians.

In this project, an arbitrary period of one year before reexamination was osen. Until the case-finding incidence can be determined accurately for the nd and subsequent rescreening period, the question of how often a test uld be done cannot be accurately answered. Previous evidence indicated at the debated lesions of intraepithelial carcinoma of the cervix may lie rmant for long periods of time. From preliminary data obtained in testing e first 14,500 women of our second screening the trend indicates a marked increase in the total number of cancers and intraepithelial lesions and a muc higher ratio of intraepithelial lesions to invasive cancers, and this finding make it seem possible that a practical and economically more feasible period fo population screening may be much more than one year.

The findings in this project have again focused attention on the predictio that the genital-smear technique has its greatest potential usefulness in intra epithelial carcinomata, or "early cancer," and in its precursors. The evidenc indicates that a high percentage of such localized disease is curable. Th investigations discussed here as a preliminary report indicate that a highl significant case-finding rate of unsuspected cancer can be accomplished i population screening by the procedure and plan used. The early detection diagnosis by biopsy, and early treatment of incipient cancer in this large teste group should change the incidence rate of clinical cancer in subsequent year Continuation of this research by the annual testing of the same populatio group should confirm this prediction and contribute to the fund of knowledg needed for the solution of the unsettled question concerning the age-specif incidence and the relation of *in situ* cervical carcinoma to invasive uteri cancer.

Summary

The plan and objectives of population screening for the detection of uteri cancer have been reviewed. The progress and preliminary results during tw and one half years have been summarized.

The women examined during the first screening totaled 95,000. Of th number, 27,000 have had a second annual genital-smear test. The grea majority, 982 of each 1,000 in the first screening, were found to be essential negative. These women could be assured there was no evidence of uteri cancer. Of the 18 per 1,000 who were advised to have biopsy for furth study, the pathologic study was completed in 15.

In 627 women (0.78 per cent of all women examined) intraepithelial ca cinoma of the cervix or invasive cancer was detected by the vaginal cytolog examination. These findings, as confirmed by tissue study, were found in t first 80,000 women examined during the first screening. A total of 374 wome (0.47 per cent), or almost 5 in 1,000 of all women examined in the first scree ing, had unsuspected disease.

The results of the first screening in this investigation clearly indicate th the genital-cytology technique can be utilized effectively in population scree ing for early uterine cancer case findings. Used as a detection or screeni procedure for uterine cancer, the vaginal smear is an excellent practical canc test.

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TEAMWORK BETWEEN PATHOLOGISTS AND CYTOLOGISTS

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Part I

By Louis S. Lapid

Many years have elapsed since Papanicolaou and Traut published thei classical monograph on cytology. Since then, innumerable articles have recorded the progress made in this field on all body surface areas. Morpholog cal cytology has become an accepted routine clinical laboratory procedure a more and more persons have become interested, and have become aware c its importance and of the excellent results obtained. A perusal of the literature showed that in 1946–1947, article after article was written on cytology Today, the occasional paper discussing this subject deals with new techniques or instruments for obtaining better results from this procedure, as abrasio brushes or smear, and cell block of secretions or body fluids.

We could make this paper one of the shortest ever presented at this academ by writing, under the title "Teamwork between Pathologists and Cytologists, the statement "Teamwork there should be, but it is not always maintained. One point must be made clear before we become involved in the assets c cytology. No doubt the cytologic diagnosis is an important tool, but th ultimate operative decision must still depend upon further evidence, be it positive biopsy, or most convincing X-ray finding. As far as malignant ne plasms of the uterus are concerned, we, at The Mount Sinai Hospital, still a here to a positive tissue biopsy before surgery or radio therapy is instigated This procedure should be followed, even by the most enthusiastic cytologis

Progress has been made in pathology, as in all fields of medicine, and w must agree that lesions once considered benign are now considered malignan The immortal Virchow's diagnostic error in the case of Frederick III, Emper of Germany, is a prime example of our realization that a lesion may be mali nant without invasion. With our present knowledge, we sometimes diagno carcinoma at a very early stage. True, we may ponder over the slides f days before arriving at a final report, or other equally competent pathologis may differ with the diagnosis, but we are all aware that we are not dealing wit something static. What may appear to be questionably benign today may malignant in two or three years.

Cytologists, on the other hand, do not deal with mitosis, invasion, or loss stratification. Their diagnosis is based on changes in the individual cell Some cytologists claim to diagnose "*in situ*" lesions as such, others go so f as to diagnose "precancerous" lesions; but the over-all general report dea with carcinoma cells present, carcinoma suspected, or negative.

How, then, can a pathologist who has spent years of schooling, of specialize training, and of clinical experience associated with his specialty react towa the neophyte who invades his domain? There must be an emotional fear a esentment on the part of the pathologist toward the cytologist. How can hese meagerly trained persons be so blunt in their diagnosis when, at times, he diagnosis cannot be made from the submitted tissue? Still, this occurs vith increasingly better results as the incidence of false positive cellular smears lecreases.

Those of us who have attempted to read routine exfoliative cytological lides have found this task to be energy-consuming and boring. In many arge institutions, the gynecologist has assumed the role of cytologist and, fter years of experience, has become proficient in his endeavor. He, in turn, as trained lay technicians, usually girls who have worked in hematology or vho may have taken a course in cytology and have been converted into pseudo-ytologists with fairly good results. Lay technicians must be controlled by a hysician-cytologist who has the knowledge of the multiplicity of pathologic onditions that might be associated with some degree of cell atypism. Certain eneralizations that the trained mind of the thinking microscopist can establish an not be drawn by the skillful but untrained lay technician. It is truly a nisfortune that the trained pathologist is shying away from the field of cyology, not only from a diagnostic or confirmatory approach, but from its pplicability for research.

In large institutions, pathologists usually do not have the time or the inlination for this study. In small institutions, they do not have enough maerial for it, or the physical facilities are absent. Not infrequently, hematoxyin-eosin stain is used, which does not give the desired results.

With our present knowledge of cancer, early diagnosis still affords the best esults from the therapy we have at our command. Many cases of early symptomatic cervical carcinoma have been detected by the vaginal smear, nd this, in turn, has led to a positive tissue biopsy. The pathologist may be lerted and request further biopsy in the presence of a positive smear and egative biopsy. Six months ago, two pregnant women had smears that vere reported as positive for cervical cancer. The biopsy had to be repeated hree and four times before the pathological diagnosis was reported as carinoma. It was not difficulty in interpretation—the correct site of biopsy vas not obtained. This point is one of the advantages of cytology, the availbility of a greater surface area. In bronchoscopy, apical lesions frequently annot be biopsied, but smears for cellular study are obtainable.

We do not believe this is the proper place for a statistical report. Still, a uick résumé of some of our results seems warranted.

In 7,000 cases, 46 vaginal smears reported as positive were confirmed by nitial biopsy; 16 cases were false negative; 3 cases were false positive; 12 ases were reported as indicating that dyskaryotic cells were present. The resence of these cells was substantiated by biopsy. It is of importance to ote that 8 per cent of these cases developed cervical carcinoma three to five ears after the initial smear and biopsy. Recently, a follow-up was made on atients who, from 1940 to 1946, presented biopsy similiar to those found in ersons who had dyskaryotic cells in their vaginal smears. There were 16 cases this group and, again, 8 per cent of these women developed cervical carcioma over a period of four to seven years after initial biopsy. Seven cases were reported as positive, and biopsy had to be repeated one to five times, ov a period of two months to three years, before a positive diagnosis of carcinom was made.

Of the 16 cases reported as false negative, 13 were adenocarcinoma of t fundus, but 10 of these 13 cases did not have endometrial aspirations. Sin the addition of the latter step, our results have improved to 87 per cent a curacy. Some institutions have reported 93 per cent correct diagnosis fundal carcinoma by cytological means.

The results of bronchial smears compared with biopsy have been goo Analysis of upper lobe lesions revealed that better results were obtained smear than biopsy.

Certain aspects in the field of exfoliative cytology must be considere Used as a diagnostic procedure in vaginal or sputum examination, cytolog has its greatest value in the screening of large groups of patients. This ma be carried out in cancer detection clinics, or in the private physician's offic A negative smear in the presence of signs or symptoms, however, should n lull the examiner into a false sense of security. Further tests must be carri out.

When considering the socioeconomics of cytology, we should give thoug to the possibility of establishing cellular diagnostic centers. These could government-operated or privately controlled. Because of the volume of wor the personnel should be well trained, and must be well supervised. The centers could serve a triple purpose: screening, diagnosing, and facilitati research.

Desquamative cytodiagnosis is not necessarily a reservation of the certifi pathologist. The conscientious gynecologist whose clinical problem deman an answer can also develop that faculty of cytodiagnosis. But tissue m croscopists must take a more active role in the field of exfoliative cytolog They are more adequately trained and, therefore, much more capable in t finer differentiation of cellular pathology. This, we feel, is especially true gastric and renal studies. In addition, the impetus provided by the train pathologist who actively enters the field of cytology will add a great deal basic research of cellular dynamics.

The relationship between the Papanicolaou test and tissue biopsy shou not be placed at opposite poles in our diagnostic sphere. One should be adjuvant of the other. At the time of initial examination, the combination smear and biopsy gives much better results than these tests give individual

PART II

By Paul Klemperer

There is very little I can add to what has been said by Doctor Lapid exce possibly, to re-emphasize some of the points he has presented. It is obvio that the period has passed in which tissue pathologists were antagonistic the idea that desquamative cytology can make a valuable contribution cancer diagnosis. This period was marred by partisanship. But the em tionalism has quieted down, and I believe tissue pathologists and cytologi have begun to work together in harmony and to develop the field of cy iagnosis cooperatively. I believe they can give each other considerable help nd stimulation. Virchow, about 100 years ago, wrote that science is large nough to accommodate every worker in its field who does not pretend that is contribution is of exclusive value. I maintain that training in exact obervation would make the tissue pathologist eminently qualified to enter the eld of cytodiagnosis, but he cannot make an exclusive claim to this field. In act, I believe he should not do so, in his own interest. With the constant rowth of laboratory medicine and with the ever-increasing demand on his ime, he is unable to cope with this expanding field of diagnostic activity. he pathologist must realize that he has to relegate this area to other laboraory associates because nothing jeopardizes diagnostic reliability more than ack of time. We tissue pathologists who are still in charge of hospital laboraories must concern ourselves seriously with how best to solve the problem of n adequate organization of the cytodiagnostic service. I believe strongly hat lay technicians should only aid, and should not be given full initiative in ancer diagnosis. It must be left to the judgment of the cytologist-in-charge o decide how much responsibility he is willing to delegate to his lay techicians.

But who shall be the cytodiagnostician-in-charge? If we had enough patholgists trained in all fields of laboratory medicine, I should vote for them as the nost desirable candidates. But we suffer a serious lack of such personnel. f we are realistic, we must consider other qualified people, and I sincerely elieve that a person trained in microscopy, whether he is a certified pathologist r a gynecologist or other medical specialist, can assume the responsibility. n fact, I believe that a person without a medical degree, providing he has eally adequate training in the recognition of cell structure, can become a cytoiagnostician. I believe that the future development of laboratory medicine emands, in general, the integration of the basic scientist into medical investigaion. Could any one be better qualified for an activity that requires attention o minute detail than the graduate in zoology, embryology, or histology? uch a worker would bring his scientific knowledge of the intimate structure of he cells to the problems of cytology, and medical investigations would profit y it. The routine duties of a cytodiagnostician are laborious and monotonous. hey must be made attractive by opportunities for serious and fundamental ytologic research. Organization of cytodiagnosis along such lines would enefit tissue pathology equally, and I believe it is the tissue pathologist who hould take the initiative in suggesting to the hospital administration the ecessity of such developments.

ACCOMPLISHMENTS AND SHORTCOMINGS OF CANCER CYTOLOGY

By Emerson Day

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If one takes a general view of the achievements of cancer cytology, the accomplishments now outweigh the shortcomings. Perhaps the most importan general accomplishment has been cytology's coming of age during the pas five years, as evidenced by the scope of the conference sponsored by the Ne York Academy of Sciences and the Damon Runyon Memorial Fund for Cance Research on which this monograph is based. The progress can be attribute to the persistence of cytologists, the understanding of many pathologists, an the accumulating experience of clinicians. The feuding that muddied th field seems to be largely over. This is good, because cytology and patholog are, in effect, two arms of the same discipline. They have a single objectiv that of determining the nature of tissue changes.

The new cooperation is best demonstrated by the Inter-Society Cytolog Council, described elsewhere in these pages by Doctor Meigs. This organization is a working coordination of pathologists and cytologists and of clinician concerned with the application of cytology to cancer control. All those wh are involved in the applications of cytology, both clinical and research, wi find that its strength lies in the close cooperation of all three segments of th team. The accomplishments of cytology can be no greater than the level competence of each part.

In this paper I shall discuss the accomplishments and shortcomings of cancer cytology as seen from a clinician's point of view, first as applied in practic and then as a research tool. In this discussion, I have not attempted a comprehensive review of the literature on cytology. It must be emphasized tha these observations represent only my own point of view, which has been developed and enriched by association with an excellent staff of clinicians, cytologists, and pathologists in the work of the Strang Cancer Prevention Clinic the Memorial Center, but members of this staff have not been consulted c what I have to say and have no responsibility for any controversial points tha may be raised in the following comments.

In evaluating the success and failure of cancer cytology in its clinical applications, it is important to distinguish two uses of cytology: the one, screenin the other, diagnosis. In effect, these two functions form a continuum, bu certain requirements of screening procedures need to be considered speciall

Screening

The basic characteristic of a screening procedure is that it is used in group of presumably healthy persons to identify those individuals who are in nee of further diagnostic attention. It may be applied to members of a communit or to selected groups of individuals where, for special reasons, there is an increased risk of disease. In cancer control efforts, such selected groups may all women, or men over 50, or those with certain environmental exposures such as oil or chromate workers or heavy smokers.

To be useful as a public health or clinical measure, a screening procedure must meet certain criteria:

(1) The procedure must be relatively simple both at the clinical and at the laboratory level. Its performance must require a minimum of time and effort on the part of the doctor or technician, and it must be simple enough to be acceptable to the asymptomatic patient. At the laboratory or reading end, it must not be costly or present great technical difficulties.

(2) The yield of significant findings must be commensurate with the effort expended.

(3) The results obtained by a screening procedure do not have to meet the highest level of diagnostic accuracy but must meet certain standards of reproducibility and reliability.

Measured against these criteria, exfoliative cytology has demonstrated a range of success and failure. Its outstanding success is the application of cell studies to the detection of early cancer of the uterine cervix and, to a lesser extent, to detection of cancer of the endometrium. Here we are dealing with a readily accessible site. The taking of vaginal and cervical smears by aspiration and swab or scrape requires no more than an additional minute or two in the course of a familiar office examination and entails no discomfort to the patient. Methods are being studied which suggest that it may be possible to obtain suitable cytologic preparations by self-administered methods that would extend further the application of this as a mass screening procedure. The uterus is the second most frequent site of cancer in women, so that the yield of significant data is high. Historically, vaginal and uterine cytology has been more intensively studied than any other, so that the reliability of cytology in this area is already high and constantly improving.

These statements can be checked against the wide experience with uterine cytology as a screening test that has accumulated in cancer centers and community programs throughout this country and abroad.[1-7] At the Strang Cancer Prevention Clinic, it is the practice to take a vaginal aspiration and a cervical swab smear routinely at the annual examination of all adult women. During the seven-year period, 1948–1954, over 91,000 screening vaginal-cervical smears were performed on nearly 28,500 women. In this group of presumably well, adult women, 84 cervical cancers and 30 endometrial cancers were diagnosed histologically for rates of approximately 3 per 1000 and 1 per 1000 respectively. At Strang Clinic, 40 per cent of the women are under the age of 40 and approximately two thirds are Jewish. In the extensive study of women in Memphis, Tenn., with a different age and racial distribution, Erickson and his group report a rate of 7.8 proven cervical cancers per 1000 women examined.[1] These figures are consistent with the expected incidence of cervical carcinoma based on vital statistics, and give further evidence that this method is a reliable screening device.

In studying the yield of any screening procedure, it is important to evaluate its specific contribution to the end result. For this purpose, 76 consecutive cases of uterine cancer diagnosed at Strang Clinic have been analyzed for the

TABLE 1
ROLE OF CYTOLOGY IN DETECTION OF 76 CARCINOMAS OF THE UTERUS
(From Strang Cancer Prevention Clinic, Memorial Center)

	Cervix	Corpus	Total
Cases detected	59	17	76
C.T.C. essential to detection	35 (59%)	2 (12%)	37
C.T.C. not essential to detection	24	15	39

role that the routine smears played in leading to suspicion of disease and final diagnosis (TABLE 1).

The clinical records were reviewed for abnormality on history or physical examination, which would indicate the need for biopsy or for dilatation and curettage. Those cases in which there was no clinical lead on history or physical examination, in other words, those in which the smear was the only evidence of abnormality on primary examination, were credited to cytology and listed as "C.T.C. (cytologic test for cancer) essential to detection." This was true in 35 (59 per cent) of the 59 cases of proved cancer of the cervix and in 2 (12 per cent) of 17 cases of cancer of the endometrium.

In this series of cervical cancers, analysis of the role of the Papanicolaou smear in relation to stage of disease at time of detection has not been made In the Memphis study, the smear was essential to detection in 60 per cent o all cervical cancers diagnosed and in 89 per cent of those detected at the pre invasive stage.[8] Similar figures have been reported in a study of 48 preinvasiv carcinomas of the cervix detected in private practice in San Diego, Calif.[4] Th demonstrated ability to detect cancer of the cervix at the "preclinical stage, when it is localized to the mucosa and curable, is an outstanding contributio of cytology to cancer control.

The relative lack of success of routine vaginal-cervical smears in detectin presymptomatic cancer of the endometrium in the Strang Clinic study (TABL 1) demonstrates the importance of the role of direct sampling for success i cytologic screening. This further emphasizes the need for improvement i methods of obtaining cell samples from within the endometrial cavity. Docto Hecht is to present a paper on this subject later during these meetings.

Another means of testing reliability of a screening method is to analyze th original test report against final diagnosis. Such an analysis for the same 7 carcinomas of the uterus is presented in TABLE 2.

There were no false negative reports in the 53 cases of preinvasive or *in si* carcinoma of the cervix, while there was 1 in the 6 cases of invasive cervic carcinoma (subsequently proved to be a reading error) and 4 misses among th 17 cases of endometrial carcinoma. It is possible that there were unrecognize cases of cervical cancer in the Strang Clinic population during the period this study (*i.e.* unrecorded false negatives), but the nature of the patient co tact and follow-up is such that this is unlikely.

False Positive and False Negative Smear. The success or failure of a canc screening test is classically measured in terms of the percentage of false positi and of false negative results. Dunn and Greenhouse have set an arbitrar

TABLE 2
CLASSIFICATION OF ORIGINAL SMEAR IN 76 CARCINOMAS OF THE UTERUS
(From Strang Cancer Prevention Clinic, Memorial Center)

	Cervix			Corpus	Total uterus
	Preinvasive	Invasive	Total		
Proved cases	53	6	59	17	76
C.T.C.					
Positive	21	5	26	7	33
Suspicious	32	0	32	6	38
Negative	0	1	1	4*	5

* Three patients were clinically suspicious and were referred for diagnostic curettage.

maximum allowable level of 5 per cent false positives and 10 per cent false negatives in early, localized cancer.[9] For an accessible site such as the cervix, Papanicolaou smears fall well within these limits when taken by a practitioner acquainted with the method and read in an experienced laboratory. Many cancer screening tests have fallen into disfavor because, while they showed an adequately high level of pick-up of advanced disease, they were not sensitive enough and gave false negatives in cases of early cancer. Studies such as those reported above for cervical cancer indicate that the screening of exfoliated cells is an effective test for cancer even at the *in situ* stage before there is any other clinical evidence of disease.

Similarly, accumulating experience indicates that the false positive, often cited in the past as a major shortcoming of cancer cytology, is a diminishing-o-essentially-nonexistent problem in experienced hands. The role of experience in reducing the frequency of false positive readings is well illustrated in Foot's study of 1000 consecutive cell studies of the respiratory tract.[10] My own feeling is that the days are over when the false positive report presents a significant problem in properly conducted cytologic screening programs. The key to soundness of applied cytology is the practitioner responsible for translation of reports into management of the individual patient. Even an occasional false positive is not dangerous until it leads to unnecessary treatment. This problem is not new, having been presented to clinicians in the past in the form of the false positive Wasserman, for example. Always the clinician's judgment must be the final determinant of action. The needless hysterectomy based on a single smear, however read and reported, damns the practitioner who decides on operation and not the science of cytology. Much of the disrepute that cytology has fallen into in the past because of "overreading" or false positives will be ruled out if the principle is adhered to that cytologic reports must be confirmed histologically whenever possible before treatment is initiated. For accessible sites such as the cervix and fundus uteri, this is always possible. For these sites, the problem of the false positive smear should no longer be listed as a contraindication to use of the method.

Finally, the success of screening cytology must be measured against the cost of the procedure. At Strang Clinic, the total laboratory costs of processing a t of vaginal and cervical smears are estimated to be somewhat in excess of three dollars. While this figure might be reduced by simplifications of screening methods, the inherent requirement for trained personnel in cytology laboratories and the associated expenses in obtaining smears may be expected to keep the cost of cytology relatively high for a screening test. Despite this cost factor, the specificity and reliability of cytologic smears and their demonstrated success in detecting *early* uterine cancer make them an indispensable part of any adequate cancer control program in women.

Before leaving the discussion of screening, I want to mention briefly the subject of the terminology used in cytologic reports. Again, it must be emphasized that I am talking from a clinician's point of view and that what I say in this connection does not apply to classifications or descriptions that cytologists use for their own laboratory or research purposes. For the practitioner, the smear report should be as simple as possible and directly translatable into management of the patient. A number of different systems have been used but, in essence, only three catagories are needed:

Cytologic repor	*Interpretation*
"Screen negative"	No special action required.
"Screen positive"	Immediate repeat smear and biopsy.
"Screen suspicious"	Repeat smear required at some stated interval.

The word "screen" does not need to be added to each report. It is included here to stress the fact that screening smears are not the equivalent of a diagnostic method. Such terminology avoids this implication and gives smear reports the significance which they really have, *i.e.* that of being indicators o the need for subsequent diagnostic procedures in those cases read as "suspicious" or "positive."

Diagnosis

I have gone into some detail concerning the use of cytology as a screening test for uterine cancer because this represents the earliest and most broadl applicable clinical achievement of cancer cytology. In certain fields, the us of cytology as a diagnostic aid in the patient who presents signs or symptom of disease is rapidly becoming of equal importance. There was a time when the term "diagnostic" had to be carefully avoided in talking of cytology because of the uncertainties and limitations of the new laboratory method. Now there are a number of areas in which cancer cytology has established its importance as a reliable diagnostic procedure in evaluation of the tumor suspect. The outstanding example is the application of cytology to the evaluation the lung-tumor suspect where the study of sputum or of bronchial washing in experienced hands can identify accurately the nature of the tumor preoperatively in from 65 to 90 per cent of cases.[11, 12, 13] This diagnostic procedure i increasingly important in the early tumor case in which the bronchoscopist i able to obtain biopsy material for histologic diagnosis in no more than on fourth to one third of instances. In effect, cytology has replaced histology the major aid to the diagnostician and the surgeon in the preoperative evaluation of lung tumors. This, of course, does not rule out the necessity for histologic confirmation at the time of operation before proceeding with definitiv surgery.

Theoretically, cytologic methods can be applied to any body surface. Indeed, cytologic studies have been made of practically all organs and cavities of the body and, for several sites, familiarity with the range of normal and abnormal cells has reached the level of diagnostic accuracy.[14, 15] There are three basic factors limiting the usefulness of cytology both for screening and diagnostic purposes that are worth reviewing in any evaluation of cytology's achievements and shortcomings:

(1) The pathogenesis and anatomic location of certain tumors is such that surface exfoliation of tumor cells cannot be expected during the critical early phases of tumor formation. An example is prostatic cancer. Even though fluid for cytologic study can be obtained on prostatic massage in most men, only in rare instances does the sample from a man without clinical evidence of disease contain diagnostic cells. Preliminary analysis of over 2000 screening prostatic smears in clinically normal men age 50 and older at Strang Clinic has yielded such discouraging results that the use of prostatic cytology is now recommended in this clinic only for the patient who presents abnormality of the prostate on digital examination. It should be pointed out, however, that prostatic smears may be extremely helpful as a diagnostic aid in evaluation of the clinically abnormal prostate, particularly when they are positive. As in all diagnostic applications of cytology, a negative report does not rule out neoplastic disease.

(2) Cells, though exfoliating in an accessible organ, may not be obtainable for smear. An example is the breast which, though a frequent site of cancer, rarely provides material for smears. When breast secretion is obtainable, cell studies may be the earliest and, often, the only way of establishing the nature of an underlying pathological process.[16]

(3) Cells, though exfoliating in abundance, are not accessible for cytologic study without undertaking procedures that are uncomfortable for the patient, and time-consuming and relatively costly for the laboratory. This last factor of relative costliness will depend upon the likelihood of cancer at the site or in the particular patient under study, and will vary in each individual case. Important examples of the usefulness of cytology as a diagnostic adjunct despite organ inaccessibility are presented by the lung and the gastrointestinal tract. Various forms of scraping, brushing, or lavaging have been developed to aid in obtaining cytologic specimens. As these are improved, reduction in the number of equivocal or false negative readings and extension of the diagnostic applications of cytology can be expected.[17]

A review of the clinical applications of cytology would not be complete without mention of its use in the follow-up study of patients treated for cancer either by surgery, radiation, or chemotherapeutic agents. Cytologic studies in these patients can increase the accuracy of treatment supervision greatly and increase the promptness of the diagnosis of recurrence if it occurs. There is increasing evidence that vaginal-cervical smears may also be of value in determining the best type of treatment, whether radiation or surgical, for patients with diagnosed cervical cancer,[18] and also for following the effect of radiation both local and distant. It is possible that this application of cytology as a measure of radiation effect may be applied to surfaces other than the vaginal mucosa, such as the buccal mucosa in men, thereby affording a broadly applicable means of monitoring radiation programs.

Laboratory Facilities

The major shortcoming of cancer cytology at present is the gross inadequac of laboratory facilities for reliable cytological services, both screening an diagnostic. I have had an opportunity during the past few years of talking with practitioners and with laboratory staffs in a number of cities throughou the country. The usual story is that an increasing number of practitioners ar convinced of the importance of cytology for earlier diagnosis and detection o cancer. These physicians would like to incorporate screening and diagnosti smears in their practice, but report that they have no place to send them for re ports that they can trust. At the other end of the problem, there are a fe laboratories that are prepared to read smears, but are receiving them in suc small numbers that they cannot keep up their proficiency even if originally wel prepared to render cytological services. While there are a number of commun ities where an effective program has been established, too often cytology i unused because it is not readily available or, if offered, is not maintaine at a reliable level.

This is a self-perpetuating situation that needs attack on a number of fronts but the sector most urgently requiring attention is that of laboratory facilitie The immediate need is for a substantial increase in the number of technician trained in screening and of pathologists experienced in cytodiagnosis. Onc staffs are available, there must be laboratories prepared to render reliabl cytologic reports at strategic points throughout the country. In order fo such a regional program to reach a satisfactory level of achievement, it wi probably require the aggressive support of such organizations as the Americ Cancer Society and the National Cancer Institute, and the guidance of a bod such as the Inter-Society Cytology Council.

A few comments are necessary in regard to laboratory staffing for cytologi services. While all diagnostic reports must be rendered by professional sta members, the scanning of screening (vaginal and cervical) smears to separat the negatives from those that are abnormal and require diagnostic reading a proper function of cytologically trained technicians. Although this role the technician has been objected to by some pathologists on the grounds th it invades the professional province, this is the only way in which screenin cytological services can be provided on an adequate volume basis. The exper ence at the Strang Laboratory of Cytology indicates that, with trained techn cians, somewhere in excess of 90 per cent of detection smears can be reliabl screened out and not require the pathologist's attention. This is an essenti saving of professional time in a busy laboratory.

Another potential staff timesaver in the attack on the laboratory bottlene is the electronic scanner or cytoanalyzer, about which we are to see more these pages. While this device still is at the research and development stag the achievement of its objective of scanning smears by instrument in lar volume would be of vital importance in enabling us to extend cytologic servic to large segments of the population. Even if the load of smears requirin uman study could be reduced by 50 per cent with electronic scanning, this vould greatly increase the number of women who could be reached by a screenng program. When one realizes that over 10,000 women die each year in he United States of carcinoma of the cervix and that most, if not all, of these ancers could be cured if detected in the preinvasive stage, the importance of etting on with this task becomes vivid.

Research

The most important research contribution of exfoliative cytology from a linician's point of view is the opportunity that it affords to study the course f tissue changes, such as metaplasias and atypias without disturbing the lesion. iopsy for histologic diagnosis has no disadvantage in the case of large lesions vhere substantial areas remain unaffected by the biopsy procedure. But vhen one is studying a lesion that may exist only at a minute focus, as *in situ* arcinoma or its antecedents, the act of biopsy may destroy or so molest the esion that its natural course, and thus its significance, cannot be determined. he study of exfoliated material gives us an opportunity to avoid this shortoming of previous methods in clinical studies of the borderline or premalignant esion. It would be extremely helpful in clinical research if the science of ytology were to become sufficiently established to replace biopsy specimens n the study of subclinical mucosal pathology. As pointed out earlier in his discussion, such a potentiality has not yet been achieved, but it must be isted as a desired goal for the research applications of cancer cytology.

To achieve such a role for cancer cytology in research and to potentiate ts usefulness in diagnostic applications we need to emphasize studies of the rigin and biologic destiny of cells. The knowledge of exfoliated cell morpholgy has been greatly advanced in the past 10 years and will continue to accumuate in the future. But we need to add understanding of the growth characeristics and potential for invasiveness, or the *biodynamics*, of cells. There is ncouragement that this dynamic aspect of cytology can be developed through iochemical studies at the microtissue, cell and subcell level, and tissue culture esearch. The addition of such a "fourth dimension" to cell studies will reatly aid in evaluation of borderline atypias, clinical management of *in situ* arcinoma, and clarification of the question of reversibility of this group of ntraepithelial lesions which have become of front-line importance in cancer esearch and control.

Summary

The accomplishments and shortcomings of cancer cytology have been reiewed from the clinical point of view. Evidence establishing the success of ytologic screening for cancer of the uterus, particularly early cancer of the ervix, has been presented. The applications of cytology to cancer diagnosis ave been reviewed, and the factors determining success and failure, both in iagnosis and screening, have been outlined.

The major shortcoming in cytology today is the acute shortage of laboratory cilities. The importance of this problem and some approaches to its solution ave been discussed.

Finally, the unique opportunities that cytology affords for clinical researc in the nature and control of cancer have been touched upon.

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Part II. Morphologic Imprints of Malignancy on Cells

CHARACTERISTICS OF MITOSIS IN TUMOR CELLS

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The malignant protoplasm of a cell of a tumor reveals no cytologically detectable structural characteristic that would distinguish it qualitatively from the protoplasm of a normal cell. The white blood cells of the circulating blood of a leukemic mouse and their normal cells of origin are structurally identical, although Furth and Kahn in 1937 found that a single cell of a leukemic mouse produced leukemia upon injection of it into a normal host. In acute human myelogenous leukemia, no qualitative structural difference has yet been detected to distinguish the circulating white blood cells of leukemia from their normal cells of origin in the bone marrow. It is not known whether leukemia cells complete mitosis in the serum of the blood in leukemia, although some do so under the microscope in saline. Hansemann in 1890 and Boveri in 1912 regarded asymmetrical mitosis as a factor in the development of a tumor cell. This is not peculiar to tumor cells, however, for abnormal mitosis does occur in normal tissue also, and some kinds of tumors possess the normal number of chromosomes with the normal form of mitotic figures. Winiwarter in 1907 believed that cells containing abnormal numbers of chromosomes were merely moribund cells. Lewis and Lewis thought that malignant cells have abnormally large centrospheres and often twice the normal number of them. According to Timonen and Therman, malignant cells may be characterized by an imbalance in the timing of the events of the mitotic cycle. The centrospheres, developing precociously prior to the ripening of the chromosomes, may multiply before the chromosomes are movable, thus producing abnormal mitosis. On the other hand, the chromosomes may mature too early before the achromatic figure is fully developed, and multiply in metaphase, resulting in daughter cells possessing more than the normal complement of chromosomes. Mitosis can be arrested in metaphase in both normal and malignant cells by administration of nonlethal doses of noncarcinogenic chemical agents such as derivatives of podophyllin or colchicine, which are powerful mitotic poisons. The mode of inhibition of mitosis is apparently the same in respect to cytological structural alteration in both normal and malignant cells (MacCardle, 1951).

Mitotic alterations in normal epidermal cells can be induced also by carcinogenic chemical agents, as observed by Biesele and Cowdry in the production of tumors following application of 20-methylcholanthrene on the skin of the mouse. Such alterations, however, may simply represent irreversible chromosomal changes to produce death-bound cells that may have nothing to do with the malignant state. Similar alterations can be induced by carcinogens without the production of tumors, and tumors can be produced without chromosomal alterations (Ludford, 1954).

The intracellular chemical and physical factors that determine the initiation of mitotic division of the malignant cell (and for that matter of the normal cell) are unknown. Mitosis is only one of the events that contribute to the formation of a malignant tumor. Studies of mitotic poisons have added little to our knowledge of the nature of mitosis in malignant cells. Perhaps extrinsic factors such as hormones and minerals may stimulate mitosis directly or indirectly in the formation of a tumor. Woolley and Little found that 100 per cent of ovariectomized CE strain mice developed carcinoma of the adrenal cortex that is a slowly-growing tumor with many mitotic figures. None of the normal mice develop such tumors. Gorbman in 1949 found malignant tumors of the anterior lobe of the pituitary gland in mice that had been radiothyroidectomized by treatment with as little as 100 μc. of radioiodine. The cells of the anterior lobe become hypertrophied and, finally, begin to divide mitotically to form a tumor, many of the cells of which possess a double Golgi apparatus. The mitotic figures of such pituitary tumors are of normal appearance as compared with those of the normal embryonic gland. The chromosomes of the hyperplastic gland, however (following treatment with radioiodine, prior to development of the tumor), are in the form of thin filaments, while those of the tumor tissue are long, thick rods of chromatin. The tumor produces, according to Furth, about 5 to 10 times as much thyrotrophin as the normal gland. Earle and Voegtlin in 1940 and Earle and Nettleship in 1943 found that normal subcutaneous tissue grown *in vitro* in tissue culture for a time will produce malignant sarcomas upon subcutaneous injection into a suitable host inbred mouse that, in turn, are transplantable *in vivo*. George Gey had found that this is so. Once developed, the malignant process is inheritable from cell to cell in mitosis. Witness the remarkable example of Jensen's sarcoma of the mouse. The malignant cells of this sarcoma, commonly carried in transplant today, are the direct descendants of the cells of the tissue that became malignant in Copenhagen 50 years ago. The mitotic figures and the number of chromosomes in the cells of Jensen's sarcoma today are the same as they were in 1903 when Jensen described them.

The malignant cell seems to recapture its embryonic capacity to initiate its own division. A. D. Mead in 1898 found that the immature egg of the annelid *Chaetopterus* formed many cytasters upon its release from the ovary into the sea water. By adding KCl to the sea water, its blocked metaphase completed its cell division to form polycytes, normally formed only after fertilization. Jacques Loeb made the brilliant discovery in 1899 that the unfertilized mature egg of the sea urchin can be induced artificially to cleave by placing it in hypertonic sea water, where it produces normal larvae by parthenogenesis. This discovery led to a long series of studies on the initiation of mitosis. Loeb found that parthenogenesis could be accomplished by treatment of the egg first with a fatty acid and then with sea water made hypertonic by the addition of $MgCl_2$. Wilson and Heilbrunn in 1952 found that the common parthenogenetic agents cause liquefaction and calcium from the cortical to the internal protoplasm of the cell, where the calcium is undoubtedly an important factor in the development of the gelation that normally precedes the formation of the mitotic spindle. The author has found that the cytoplasm of mitotically dividing cells of the early (three-day-old) slowly-growing transplant of sarcoma 37 is poorly argentophilic in preparations of tumors fixed in DaFano's fluid and impregnated with silver, whereas that of the older (six days postinoculation) highly-vascularized, rapidly-growing transplant tumor is richly argentophilic. The nature of this argentophilic material is not known. Preliminary transplantation experiments by the author revealed that the inoculation mash in a series of 50 three-day-old sarcoma 37 tumors grows to form tumors of palpable size much more slowly than the inoculum mash of an equal number of six-day-old tumors. Tumor mash consisting of approximately equal portions of three-day-old and six-day-old tumor cells inoculated into the thigh muscles of mice evidently produces more rapid growth. The structural form of the mitotic figures of these transplanted tumors of different age is apparently the same, and it is no different in tumors derived from the mash of mixed tumor cells.

The speed of mitosis in sarcoma 37 is not precisely known, and the mitotic time has not been determined at different ages of it. The transplant sarcomatous tumor was derived in 1906 from an adenocarcinoma of the epithelial tissue of the mammary gland of a mouse. In 1918, it began to change its form to the sarcomatous pattern, which still persists. Hence, the significance of its speed of mitosis in relation to the speed of mitosis in normal cells would be difficult to determine. It is not easy to recognize the prophase of mitosis in sarcoma 37.

In conclusion, it may be said that there is little cytologically detectable structural form to characterize the mitotically-dividing cells constituting tumor tissue, and none by which to distinguish a single isolated mitotically-dividing malignant cell.

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CHROMOSOME ANOMALIES IN HUMAN NEOPLASMS WITH SPECIAL REFERENCE TO THE MECHANISMS OF POLYPLOIDIZATION AND ANEUPLOIDIZATION IN THE HELA STRAIN*

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In so far as chromosomal anomalies are considered, there does not seem to be a qualitative difference between malignant and nonmalignant tissues. Quantitatively speaking, however, neoplasms usually show much higher frequencies of mitotic abnormalities and, consequently, more chromosome aberrations. Although it is misleading in part to place these abnormalities in distinct and separate classifications, three major groupings may be made; namely, variations in chromosome morphology, variations in gene arrangement, and variations in chromosome number. These may overlap to some extent.

Morphological variations include the changes of the karyotype, of spiralization systems, and of nucleic acid charge, which may produce stickiness of the chromosomes and extraordinarily thick or thin chromosomes. In mouse tumors, Levan and Hauschka (1953) found unusually contracted chromosomes in the DBA lymphoma, an extremely large chromosome in the MC1M sarcoma, and minute chromosomes in the sarcoma strains MC1M, MC1A, and S37.

Changes of the centromeric positions have been reported by several authors. Thus Levan and Hauschka (*l.c.*) found biarmed chromosomes in the 15091a carcinoma and chromosomes with a small second arm in several tumors of the mouse. The Makino group claimed to find large biarmed chromosomes in rat tumors, notably the Yoshida sarcoma (Makino, 1952a, b; Makino and Kanô, 1953). Although Makino and Hsu (1954) later found several pairs of biarmed chromosomes in normal rat tissues, the original finding that certain rat cancers have characteristically altered karyotypes may still be true for, in normal rat tissues, no such large V-shaped chromosomes have been found.

Only scanty information on human material is available. Koller (1947) found sticking chromosomes, overcondensed chromosomes, and fragmentation of chromosomes in a number of human carcinomas. Hsu (1954a) also found extensive fragmentation of chromosomes in a carcinoma. Little is known, however, about the karyotypic changes of the human neoplasms, since the chromosome constitution of normal cells has itself not been fully described or understood.

Structural changes mainly concern the rearrangements of the gene alignment. It is almost impossible to detect inversions and translocations in somatic cells other than in the giant chromosomes of Diptera unless the inversion involves the centromere or the translocation is highly unequal. Small deficiencies and

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* Supported in part by a grant-in-aid from the American Cancer Society, New York, N. Y., upon recommendation of the Committee on Growth of the National Research Council, Washington, D. C., administered by C. M. Pomerat, and by Research Grant D. R. 269A from the Damon Runyon Memorial Fund for Cancer Research administered by T. C. Hsu.

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duplications are also not demonstrable, but one can reasonably assume the exist because of fragmentation and false bridges in cancer cells. In mous ascites tumors, Tijo and Levan (1954) found definite evidence of dicentr chromosomes, "triradiate" chromosomes, and ring chromosomes.

Numerical variations of the chromosomes constitute the most easily approached anomaly with respect to obtaining quantitative data. Extensiv chromosome counts have been made in many mouse and rat tumors. These counts may be slightly biased, since the cytologist tends to select the cells tha give the clearest chromosome picture, cells that probably contain lower chromosome numbers. The differences between normal and neoplastic tissue however, are obvious. Cancer tissues generally contain more polyploids an aneuploids. Furthermore, the neoplastic cells show a much wider spectrum of aneuploidy than cells of nonmalignant tissues. In human tumors, only few such studies are known, but the results are similar to those obtained from experimental animals. The data of Koller (1947) on an adenocarcinoma the rectum showed that this cancer was of subdiploid or even hyperhaploid nature. A melanoma studied by Hsu (1954a) was found to be a near-diploid and, in the same work, a sarcoma was seen to have a very variable pattern chromosome numbers. The strain HeLa, derived from a cervical carcinoma seems to be a hypotetraploid (Hsu, 1954c). Support for this was found b Petrakis (personal communication to TCH) in terms of the measured DN content per nucleus in the HeLa strain.

Many studies of cell populations have been made that bear on the genera problems of aneuploidy and polyploidy, but little has been presented as ye involving direct observation and experimentation with living cells. The informational advantages of using living cells studied by phase microscopy an ciné techniques have been pointed out by many workers. The obvious superiority of a dynamic picture of cell division over the static one has been emphasized by Hsu (1954b). Many time-lapse, ciné sequences of mitoses in th HeLa strain have been accumulated in our laboratory and, from this materia studies have been made concerning the mechanisms of polyploidization an aneuploidization. The factors involved are as yet only partly understood, bu it is hoped that our findings and tentative ideas will be of interest to cytologists

Polyploidization

Since polyploid cells have been found to occur in nearly all the tissues c many animals and plants, the mechanisms of their formation have been theorized by various workers, and terms have been devised to describe the man variations. The classical case of endomitosis in Gerris (Geitler, 1937) ha been found in a number of organisms with various modifications. The "endomitotic cycle" may be complete with the chromosomes condensing and decondensing in a manner equivalent to the regular mitotic cycle (*e.g.*, Biesele *et al.* 1942; Levan and Hauschka, 1953), or the chromosomes may be only slightl condensed, as in early prophase, but fail to go beyond that stage (Painter an Reindorp, 1939). Increase of ploidy may take place during interphase wit no nuclear changes whatever showing, save doubling of the nuclear volume

This type of polyploidization is apparently very common and may happen repeatedly, giving rise to very high polyploid cells. For instance, the nuclei of the ileum of mosquito larvae can reach enormous sizes without any sign of mitotic or endomitotic condensation and, by the time of metamorphosis, these cells begin to show large numbers of chromosomes (Berger, 1938). In mouse ascites tumors, Levan and Hauschka (*l.c.*) found cells in mitotic stages showing groups of two, four, eight or more homologous chromosomes, indicating the doubling of the chromosome number during previous interphases. These authors suggested a term, endoreduplication, to denote such processes.

Since the cytological effects of colchicine have become known, mitotic figures in untreated tissues resembling those showing colchicine effects, *i.e.*, inhibition of the spindle and reconstitution of the chromosomes into a nucleus without anaphase, have been called "C-mitotic." This process differs from endomitosis in that in C-mitosis the mitotic course goes beyond prophase whereas, in endomitosis, all the observed phenomena occur within the nuclear membrane. With a defective spindle and the lack of cytokinesis, the chromosomes, when telophase ensues, form a nucleus of higher ploidy.

In the cells of the HeLa strain, it was noted that endomitosis was not particularly common (Hsu, 1954c). Perhaps some of the polyploidization in HeLa is due to endoreduplication. It is thought to arise primarily, however, from a variety of aberrations of true mitosis, including C-mitosis.

Sequence 8 XI R_1 contains a cell that entered mitosis and reached metaphase, but the chromosomes later reformed a nucleus without division. The chromosomes, however, did not widely disperse, as they usually do in C-mitosis. Unfortunately, this sequence was taken with an 8-mm. objective, and no detailed account can be made. Sequence 28 X L_2 shows a cell of similar behavior. The cell in sequence 8 XI R_2 provides a better demonstration of the formation of polyploidy at metaphase. Selected frames of this sequence are reproduced in FIGURES 1 to 8. This cell was noted at metaphase with a seemingly normal chromosome plate and an image of a well-formed spindle (FIGURE 1). Approximately an hour later, however, the chromosomes began to clump together, and the spindle seemed to be degenerating (FIGURE 2). As the degeneration of the spindle proceeded, the chromosomes gradually lost their metaphasic arrangement (FIGURE 3). With no anaphase, cytoplasmic cleavage began as the chromosomes showed definite evidence of despiralization, indicating telophase (FIGURES 4, 5, 6). The cleavage furrow did not cut the cell very deeply, and the process of cytokinesis regressed. The chromosomes, after more than two hours of reconstitution (FIGURE 7), formed a number of small nuclei (FIGURE 8).

The above sequence demonstrates several interesting points. First, polyploidization at metaphase may begin with a normal spindle, at least superficially without defects, and the spindle may then degenerate after some duration of metaphase. Therefore, it is not exactly comparable to C-mitosis. Second, the process requires a much longer time than a regular division (*cf.* Moorhead and Hsu, in press). From metaphase until the end of the sequence, at which time the cell was not yet completely flattened as in ordinary interphase, 6.5

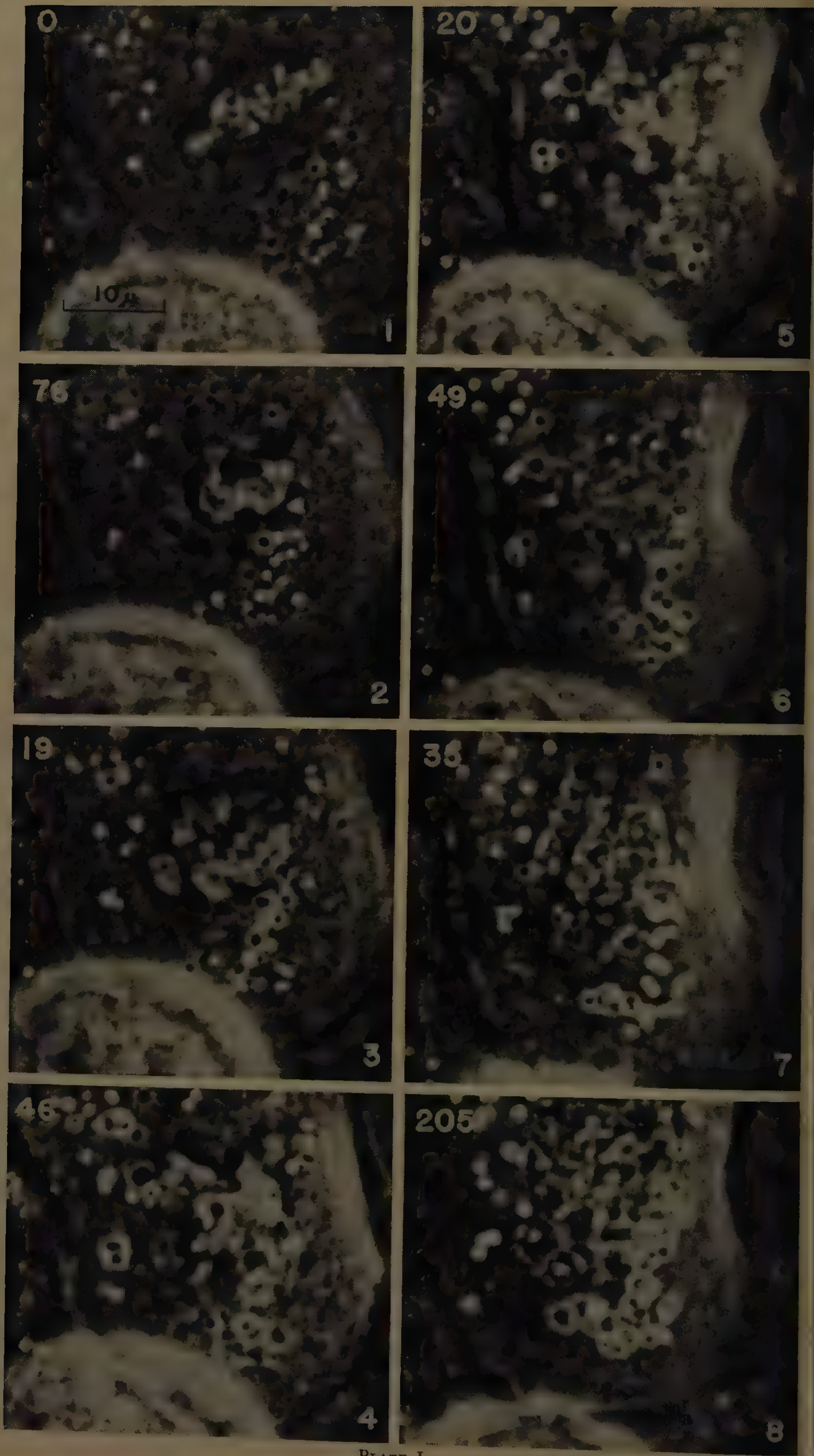

PLATE I

hours elapsed. Third, by studying stained HeLa cultures, one can often find heavily stained cells with many small nuclei or with a large, lobated nucleus and several other small nuclei. It is very possible that these cells represent the later stages of the type of polyploidization just described. If the process is similar to that of the cell in sequence 8 XI R_1, the resulting cell would have only one nucleus, and it would be difficult to determine if it represented a stage of polyploidization from fixed slides. But it is apparent that a certain proportion of the chromophilic, rounded cells does represent the later stages of metaphase reduplication.

Polyploidization of a cell can be achieved even after the chromosomes have entered anaphase. Sequence 71 VIII L_3, whose selected frames are reproduced in FIGURES 9 to 16, illustrates such a unique process. The cell was at metaphase when the filming was begun (FIGURE 9). After approximately half an hour, anaphase occurred, and the chromosomes moved to the poles in a regular manner (FIGURES 10, 11). During late anaphase, the two chromosome groups were well separated, and the cleavage process started. A constriction of the cell body at the equator and cytoplasmic bubbles that usually occur during cleavage can be noted in FIGURE 12. Suddenly, the two groups of chromosomes moved toward the center of the cell. Comparing the distance between the two chromosome groups in FIGURE 13, which represents the cell three minutes later than in FIGURE 12, it is obvious that the chromosomes have moved back. FIGURE 14 shows the two chromosome groups even closer to each other and, in FIGURE 15, they have almost mingled. Meanwhile, the cleavage furrow has receded, and the cytoplasmic bubbling has disappeared. After the chromosomes uncoiled, a single nucleus resulted (FIGURE 16).

It can be added here that unipolar spindles should be suspected as a possible cause of polyploid cells. As reported by Hsu (1954c), there is a small fraction of mitotic cells that possess a unipolar spindle. In one living cell that was followed with still photography, however, an apparent unipolar condition with all the centromeres pointing to one pole and all the chromosomes lying at one end of the cell became bipolar after the chromosomes had moved to the center of the cell. The cell finally divided regularly. Whether all unipolars behave this way is not certain.

Even when cleavage is nearly complete, the filial elements may still reunite into a single cell. This behavior has been observed in the HeLa strain by us (*e.g.*, sequence 59 IX R_1) and in rat tumors by Nakahara (1953) and Makino and Nakahara (1953). It is probably unreasonable to assume that, in such cases, the daughter nuclei reunite after their membranes have formed. The result of this would be a multinucleated cell. Nevertheless, if cleavage is completed prior to the formation of the nuclear membrane, which has been observed in some regular divisions of the HeLa cells, the reunion of such cells may give rise to a single polyploid nucleus.

Selected frames from sequence 8 XI R_2 showing the process of metareduplication. The time lapse (in minutes) between successive frames is given at upper left corner of each photograph. For magnification see scale on FIGURE 1.

FIGURE 1. Metaphase, bipolar arrangement.
FIGURES 2 to 4. Process of spindle degeneration.
FIGURES 5 and 6. Telophasic uncoiling of the chromosomes without anaphase. Note cleavage process.
FIGURE 7. Beginning formation of nuclear membranes.
FIGURE 8. Nuclear membranes have been formed. Note small nuclear vesicles.

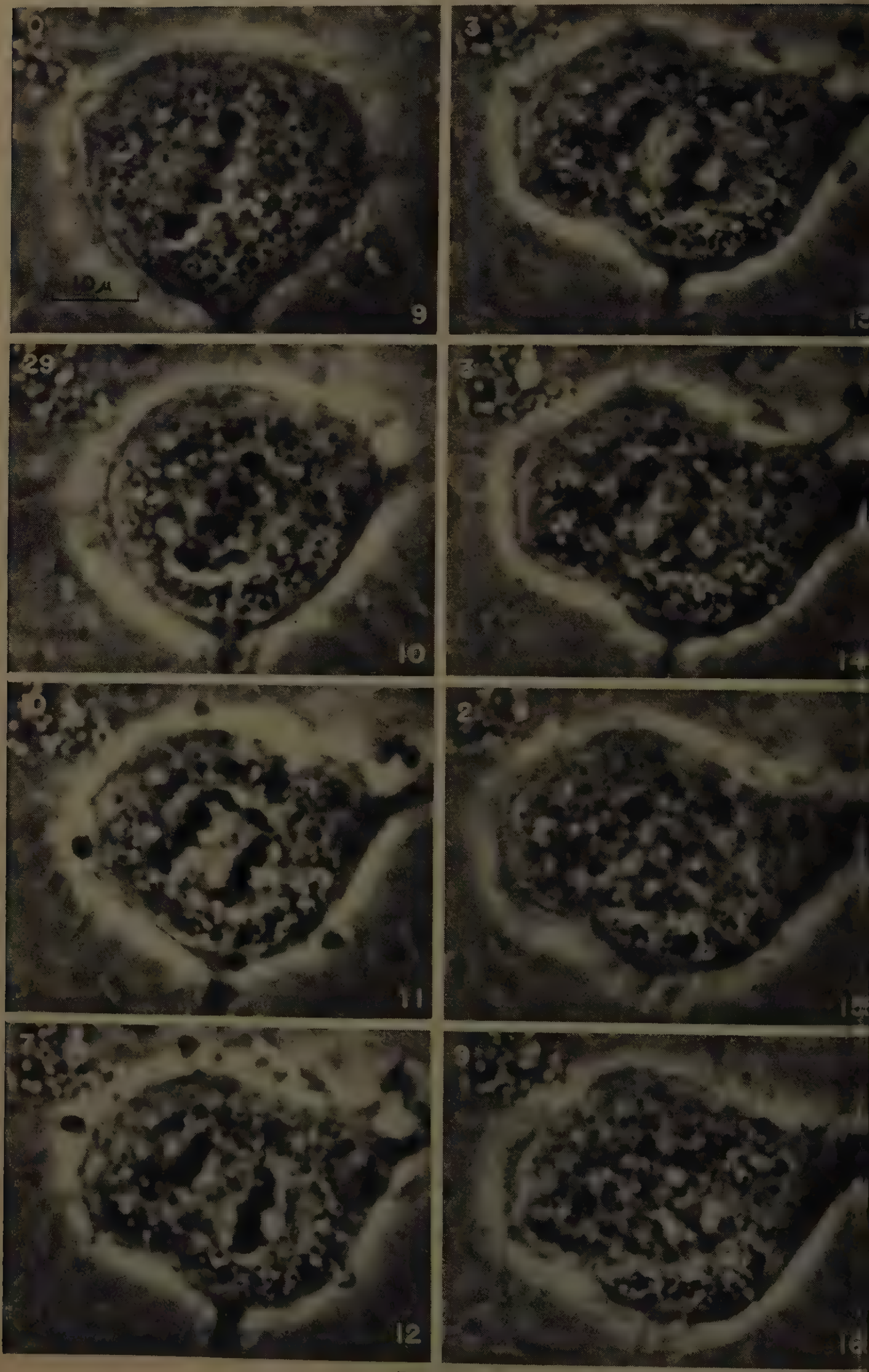

PLATE II

From the observations on endomitosis, C-mitosis, and other systems of polyploidization by previous workers and from what we have learned in analyzing our ciné records of the HeLa strain, it is felt that the mechanisms of polyploidization can best be classified according to the mitotic stage at which departure from the regular process occurs. It is desirable to use terms that facilitate categorizing the many variations. Hence, we adopt the term "reduplication" from Levan and Hauschka. There are, then, five types of reduplication, each of which produces a polyploid daughter cell:

(1) *Inter-reduplication.* This is the same as the endoreduplication of Levan and Hauschka. The polyploidization process occurs during interphase. As the reduplicated cell enters mitosis, diplochromosomes may be observed.

(2) *Pro-reduplication.* Most cases described as endomitosis fall in this category. There are certain nuclear phenomena and, in some cases, there may be a cycle of chromosome condensation, but all events take place *inside* the nuclear membrane.

(3) *Meta-reduplication.* In this we have the C-mitosis type of reduplication. Actually, C-mitosis involves a metaphase stage without a spindle. Perhaps the cell lacks a spindle apparatus to begin with, hence the chromosomes become dispersed in the cytoplasm. In the HeLa strain, metareduplication may begin with a well-formed spindle, but later the spindle degenerates and reconstitution follows with anaphase omitted.

(4) *Ana-reduplication.* Our unique instance in which the chromosomes moved back to the equatorial area from the poles during late anaphase and reunited to form a single nucleus constitutes the only case known to us.

(5) *Telo-reduplication.* This is the simple fusion of daughter cells during or after cytokinesis. Probably, all such cases of reunion produce multinucleated cells. The end result is identical to failure of cytokinesis.

Aneuploidization

There are various abnormalities of mitosis that may cause aneuploidy in the daughter cells. In nonmalignant tissues, somatic aneuploidy is at least common in mammals (Beatty, 1954), and it has been postulated by Hsu and Pomerate (1953) that some aneuploid cells may be due to nondisjunction. With so many kinds of mitotic abnormalities in the HeLa strain, nondisjunction undoubtedly occurs more often than in the nonmalignant tissues. It is most difficult, however, to obtain direct cytological evidence of nondisjunction except in extreme cases of asymmetrical division. In our motion picture records, several obvious examples of unequal division were noted. Lagging chromosomes during anaphase may be another case of aneuploidy, but they

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Selected frames of sequence 71 VIII L_3 showing the process of anareduplication. The time lapse (in minutes) between successive frames is given at upper left corner of each photograph. For magnification see scale on FIGURE 9.

FIGURE 9. Metaphase.
FIGURE 10. Early anaphase.
FIGURE 11. Anaphase.
FIGURE 12. Late anaphase.
FIGURE 13. The two chromosome groups have begun to move back toward the equatorial area.
FIGURE 14. The two chromosome groups have moved further toward the equator.
FIGURE 15. The two chromosome groups have almost mingled in the equatorial area.
FIGURE 16. Formation of a single nucleus after telophase.

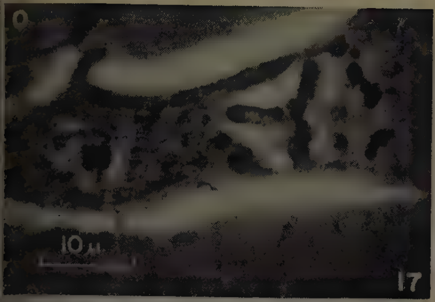
are not very common in HeLa. In several ciné sequences, chromosomes lyin off the equatorial plate may go to the poles before general anaphase, or ma lag after the majority of the chromosomes have reached the poles but, in a these cases, the odd chromosomes eventually joined one of the daughter group When the laggards were chromosomes instead of chromatids, this abnormalit must have produced aneuploidy.

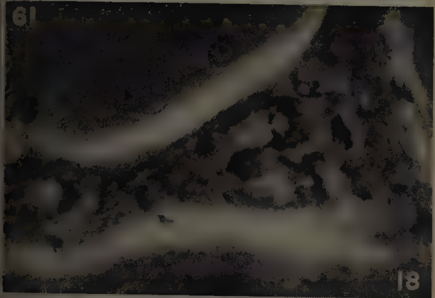
It should be of value to add that the "d-mitosis" of Tijo and Levan (*l.c.* also may produce aneuploidy, for the appearance of their d-metaphase i identical to that of a cell in normal human gingiva in tissue culture. This i described by Hsu (1955) as a case where the chromosomes finally formed nucleus, but the nucleus was divided by cytokinesis.

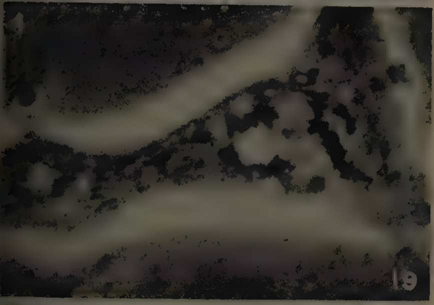
Probably the most obvious anomaly involved in producing gross aneuploid is the multipolar spindle. In nonmalignant tissues, this is observed sporad cally but, in cancer tissues, its frequency may be exceedingly high. In th HeLa strain, for instance, the frequency of multipolar spindles is about 35 pe cent of the population of the dividing cells during the first few days of subculture, decreasing to approximately 10 per cent after a week of incubatio (Hsu, 1954c). Our motion pictures have shown that a number of multipola spindles arose from simultaneous division in multinucleated cells. In othe words, a multipolar spindle is the result of incomplete fusion of two or mor separate spindles. Mononucleated cells, however, have been noted to develo multipolar spindles occasionally, and bipolars may change to multipolar FIGURES 17 to 24 illustrate an example of a tripolar mitosis (sequence 61 XI L_1), the most common type of the multipolars. FIGURE 17 shows its metaphas at side view, and FIGURES 18 and 19, early and late anaphase respectively Cleavage began as the chromosomes started their telophasic uncoiling proces (FIGURE 20), and soon three daughter cells, two large and one small, wer formed (FIGURE 21). The two larger ones entered interphase first (FIGURE 22 and 23), but the minor element reached its interphase only after a long reconstitution period (FIGURE 24).

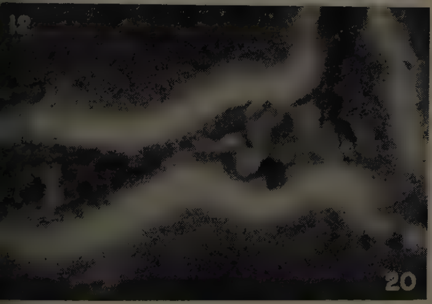
All our ciné records of tripolar and tetrapolar spindles show completion o the division. In stained preparations, multipolar spindles of higher order ar not uncommon, yet only a few cases of pentapolars are found in stages beyon metaphase. Most of the high multipolars seem to contain relatively smal and defective spindles that occupy only the central part of the enormous cell A single motion picture sequence (20 XII L_5) is available in which a multipola spindle of high order was followed. In this cell, anaphase did take place, bu the chromosomes did not move beyond the edge of the rounded spindle apparatus, and there seemed to be no spindle elongation. Cleavage occurred at th edge of the cell, but did not cut inward to any extent. The result was a gian cell with numerous nuclear bodies. Actually, this type of abnormality give rise to polyploids instead of aneuploids.

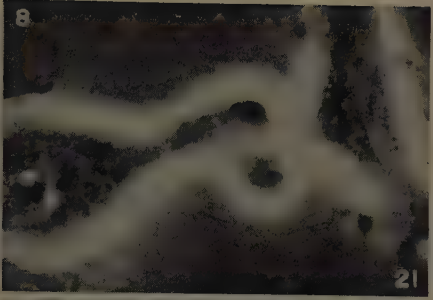
Of special cytological interest in connection with the production of aneuploid in the HeLa strain is a type of mitotic cell, which seems to remain in metaphas indefinitely. The general characteristics of such cells are: (1) they are usuall spherical, highly refractive under phase optics, and highly chromophilic i

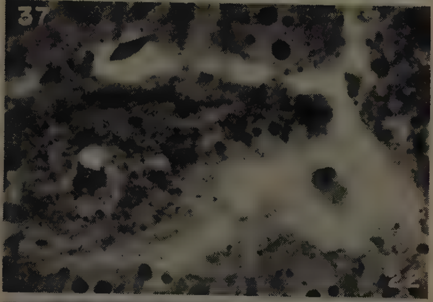


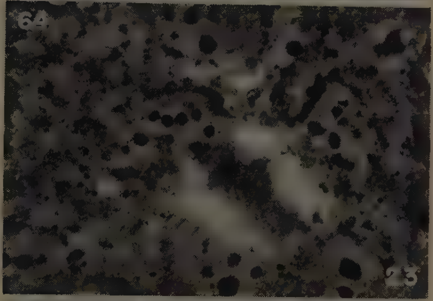












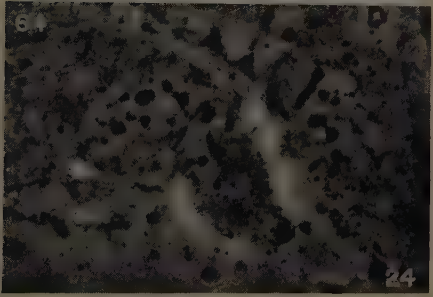
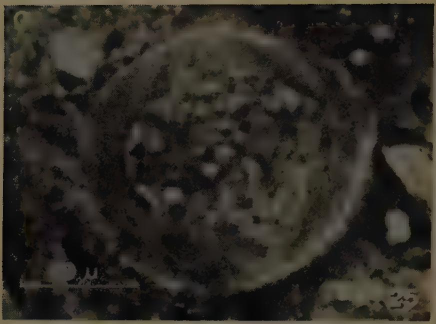
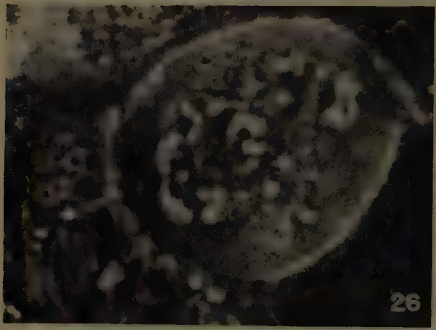


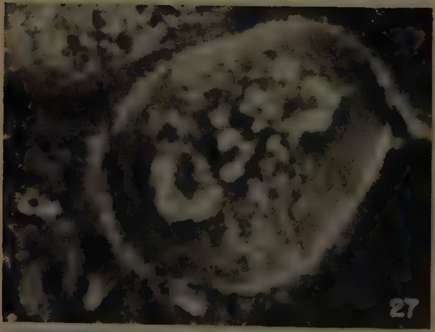
PLATE III

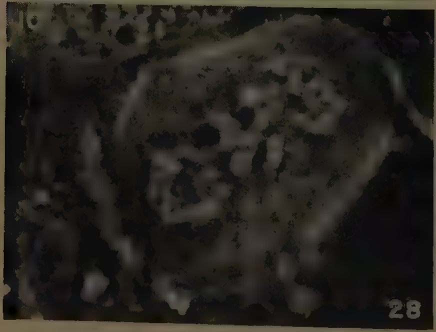
Selected frames of sequence 61 XII L_1 showing the division of a cell with tripolar metaphase. The time lapse in minutes) between successive frames is given at upper left corner of each photograph. For magnification ee scale on FIGURE 17.

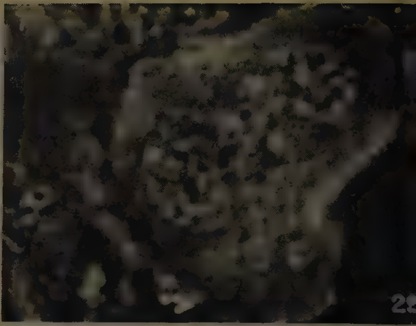
FIGURE 17. Metaphase.
FIGURE 18. Early anaphase.
FIGURE 19. Late anaphase.
FIGURE 20. Telophase.
FIGURE 21. After cleavage.
FIGURE 22. Reconstitution.
FIGURE 23. The two larger daughter cells have reached interphase.
FIGURE 24. The small daughter cell has reached the end stage of reconstitution.

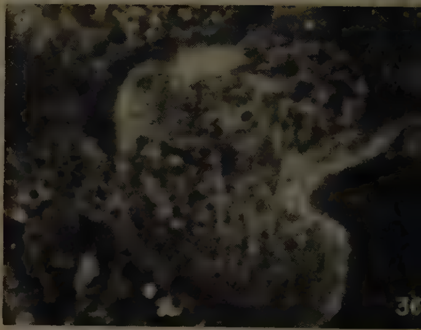


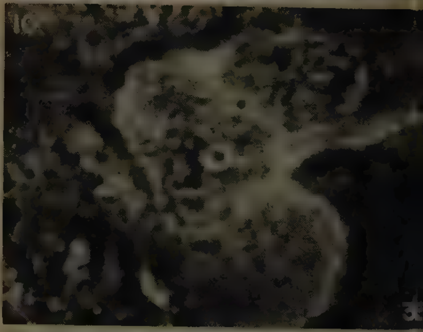












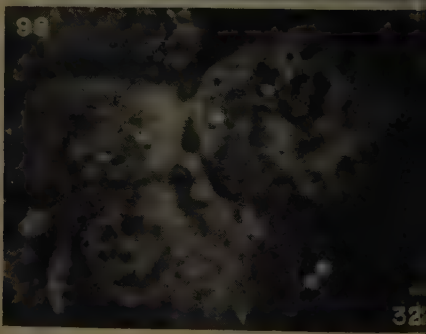


Plate IV

Selected frames of sequence 16 XII L_1 showing the division process of a malspindle cell. The time lapse
minutes) between successive frames is given at upper left corner of each photograph. For magnification
scale on FIGURE 25.

FIGURES 25 and 26. Metaphase, showing typical feature of the cell.
FIGURE 27. Beginning of telophase without anaphase.
FIGURE 28. Three nuclei have been formed
FIGURES 29 to 31. Cleavage.
FIGURE 32. Three daughter cells have been formed.

stained preparations; (2) the spindle apparatus is defective, being round, oval, elliptical, or bent; (3) the chromosomes are generally overcondensed and are widely scattered along the spindle; and (4) cytoplasmic granules (including mitochondria) are usually clumped, lying at one side of the cell. In Hsu's previous report, he described such cells from stained material (*cf.* his Figure 4, 1954c) and speculated that these cells would either divide or reconstitute into a polyploid nucleus. A third possible fate was overlooked, the death of such cells.

Most of these cells, when first located, are in metaphase. This is not surprising, because their metaphase usually lasts for many hours. During this stage, the chromosomes move actively, but the movements of the chromosomes are not synchronized, thus giving a scattered appearance. FIGURES 25 to 32 show how one such cell (sequence 16 XII L_1) divided. FIGURES 25 and 26 show the metaphase with defective spindle, dispersed chromosomes, and aggregated cytoplasmic particles. The chromosomes began to despiralize while they were scatterred over the spindle (FIGURE 27) and, during telophase (FIGURE 28), three main nuclei were formed. The cleavage process (FIGURES 29, 30, and 31) divided the cell into three daughter elements (FIGURE 32).

Among 39 such cells, which were followed until they passed the stage of the long metaphase, 20 finally divided, 18 died, and only 1 reconstituted, as in metareduplication.

The origin of such cells (it is suggested that they be termed "malspindle cells") was partially solved by two motion-picture sequences. Both began at metaphase with a seemingly perfect bipolar spindle. In sequence 62 XII L_1, the chromosomes gradually spread out and the spindle lost its definition and became somewhat rounded. In sequence 4 XIII R_8, the bipolar spindle split lengthwise, yielding three poles and a split metaphase plate. Later, this spindle also became round with the chromosomes scattered. The cell in the latter sequence eventually divided into three daughter cells in a manner typical of malspindle cells.

It is of interest to note that certain atypical mitoses in a rat sarcoma, reported as early as 1913 by Lambert, showed an appearance and behavior extremely similar to those of the malspindle cells. Lambert, however, regarded them as multipolars.

It is now of prime importance to determine which cells, if any, derived from such various abnormal divisions can survive for long periods of time, and to learn if they participate in the cell lineage of a given cancer. It is hoped that the use of a long-term culture chamber designed by Rose (1954) may provide the needed information. In this chamber, cultures can be maintained, and photomicrographs can be made simultaneously for two months or more without subculturing. Data collected from such studies may throw light on understanding the nature of cell division and the problem of somatic aneuploidy.

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NUCLEOLAR VARIABILITY IN HUMAN NEOPLASTIC CELLS*

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Since 1896, when Pianese first noted that nucleoli were larger in tumor cells than in normal cells, numerous investigators have reported observations on nucleolar characteristics and differences in neoplastic tissues and in their normal or benign analogues. Nucleoli in both animal and human tumors have been described as larger, more numerous, and more irregular in shape and distribution in malignant tissues. Excellent reviews and analyses of nucleolar characteristics have been published by Gates[7] for normal tissues and by Biesele[1] for tumor tissues.

The observations on nucleolar variability in human neoplastic cells presented in this paper are based on nucleolar studies in gynecological tumors. Previous reports in the literature on nucleolar analyses in malignant and nonmalignant tissues of these tumor types were based mainly on ratios derived from nuclear-nucleolar measurements. Von Haam and Alexander,[18] in a biometric study of benign and malignant cells, concluded that nucleoli increased in number and relative size in malignant nuclei. Page and MacCarty[15] determined that nucleoli were larger in malignant ovarian tumors, since the nuclear-nucleolar ratio was significantly smaller in malignant neoplasms than in nonmalignant. Similar conclusions were drawn by Naidu[14] and by McCormack[13] for uterine nucleoli.

Basic Concept of Present Work

The nucleolar studies presented in this paper have been done as part of a group project that involves the analysis of gynecological tumors of different histological grades of malignancy by cytochemical, biochemical, and isolated cell technical methods. The tumors under investigation are obtained in the operating rooms of the Presbyterian and Francis Delafield Hospitals as soon as they are surgically removed. Pertinent gross pathological data are noted and, usually within 15 minutes after the specimen is received, separation of representative regions of the tumor is underway for distribution to the several project laboratories.

Histological Grading

Sections of multiple areas of each tumor have been graded histologically by microscopically analyzing hematoxylin and erythrosin preparations. Two investigators graded the sections first, separately, and then in consultation to derive the grade of each tumor.

The papillary cystadenocarcinomas of the ovary and endometrial carcinomas

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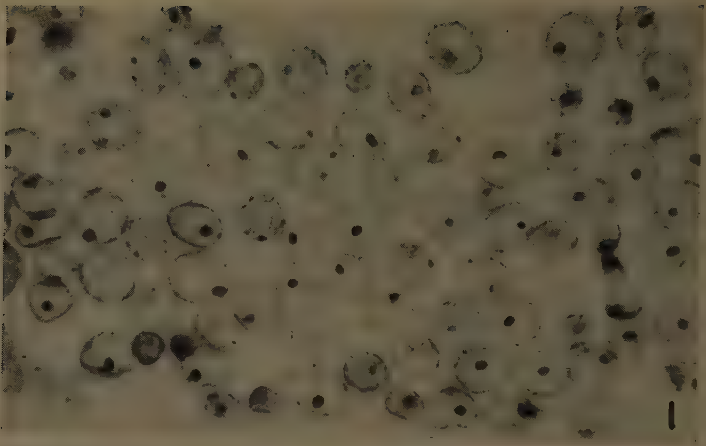
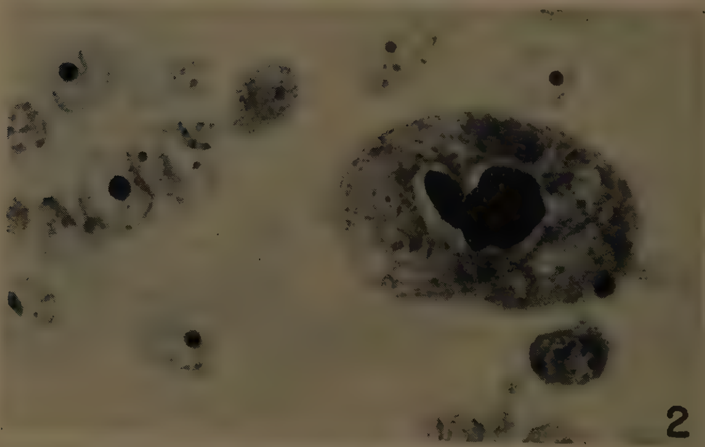
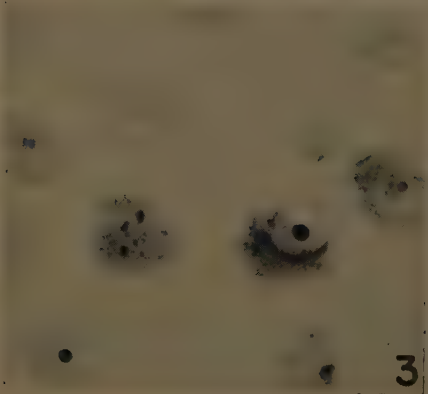
* This investigation was supported in part by grant No. 215 from the Damon Runyon Memorial Fund, New York, N. Y., and by grant No. C-2428 of the National Institutes of Health, Public Health Service, Bethesda, Md.

† The authors wish to thank Doctor Emanuel Friedman for aid in the statistical calculations and analyses.







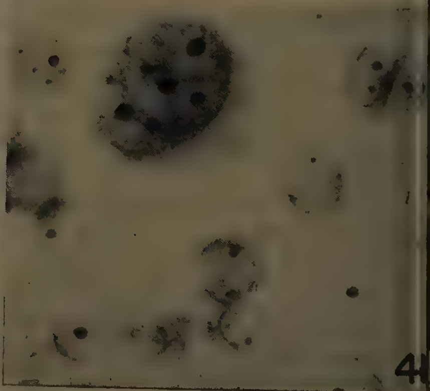


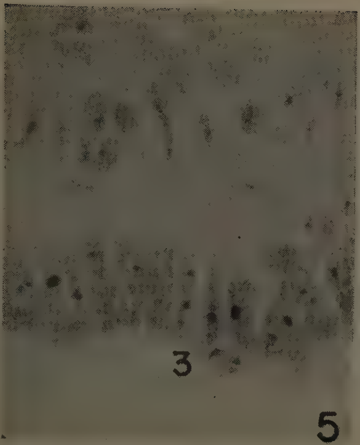
PLATE I

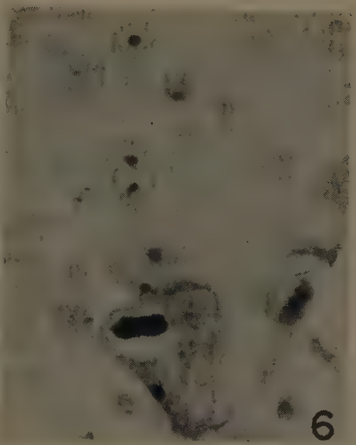
All photomicrographs on this and succeeding plates were taken at the *same magnification*. All were pro essed by the methyl green-pyronin Y method.

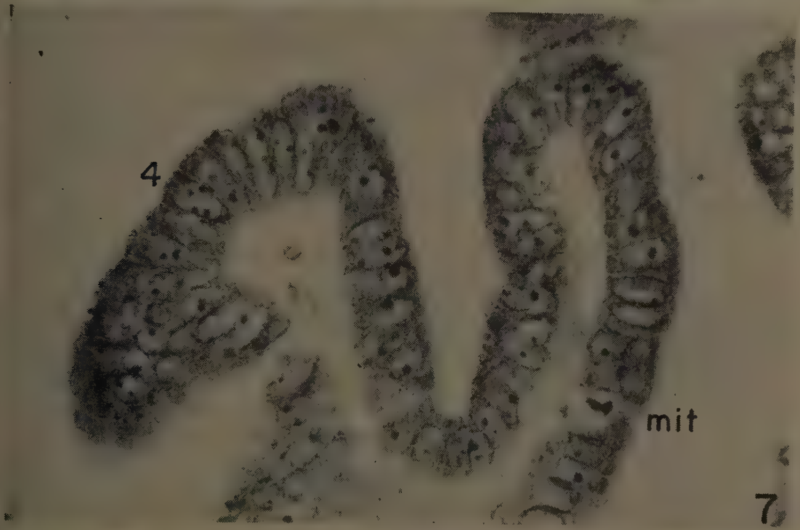
FIGURE 1. Ovary: papillary serous cystadenoma. Benign tissue smear. Note the relatively small, singl rounded, or oval nucleoli. ×918.

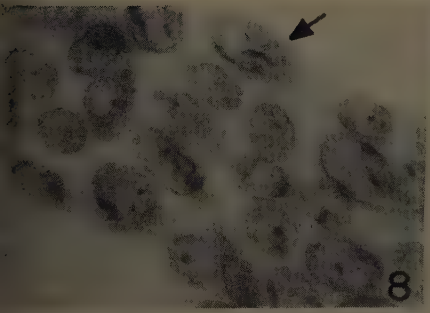
FIGURE 2. Ovary: papillary serous cystadenocarcinoma. Grade III tissue smear. Compare size of sing nucleoli with those of benign case of FIGURE 1. Note irregular form of massive nucleolus in nucleus at right figure. Some nuclei contain multiple nucleoli of varying sizes. ×918.

FIGURES 3 and 4. Ovary: papillary serous cystadenocarcinoma. Grade III Ascites fluid smear from sam case as FIGURE 2. Single large nucleoli, similar in size to those of tissue cell nucleoli. FIGURE 3 shows a nucle with eight nucleoli (not all on plane of focus). FIGURE 4 shows a binucleate cell: nucleus in focus with four lar nucleoli; nucleus not entirely in focus with eight nucleoli. ×918.









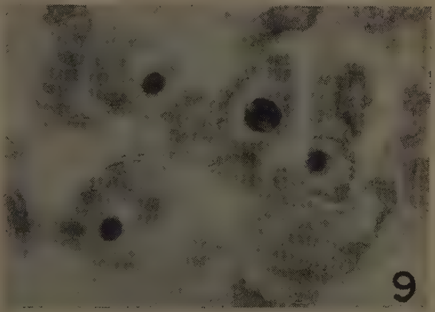


PLATE II

FIGURE 5. Ovary: papillary serous cystadenoma. Benign section. Single nucleoli as in tissue smear of IGURE 1. Note nuclei with two and three nucleoli. ×918.

FIGURE 6. Ovary: papillary serous cystadenocarcinoma. Grade III section. Compare nucleolar sizes with hose in FIGURE 5. Note large double nucleolus. ×918.

FIGURE 7. Ovary: papillary serous cystadenocarcinoma. Borderline section. One to four nucleoli per ucleus. Metaphase division present. ×918.

FIGURE 8. Ovary: papillary serous cystadenocarcinoma. Grade I tissue smear. Arrow indicates elongated ucleolus. ×918.

FIGURE 9. Ovary: papillary serous cystadenocarcinoma. Grade III section. Large single nucleoli, one evi-encing vacuolization. ×918.

have been selected for primary analysis in this project because they gro
relatively freely into a cavity, either a cystic cavity of the ovary or the endo
metrial cavity, and thus their growths are less modified by their organ matri
tissue than tumors that primarily invade stroma. Invasive malignant tumo
such as cervical epidermoid carcinomas present a more difficult problem f
histological grading because their original tumor architecture is often obscure
for clear-cut evaluation by the invaded connective tissues.

Cytochemical Aspect of This Project

The cytochemical phase of this project has concerned itself with the localiza
tion of specific cellular substances in tumor cells and with observations on bo
visual and measurable differences in these chemical constituents in relation
the several degrees of differentiation from the normal or benign tissue of orig
of the malignant cells. One of the cellular constituents cytochemically ana
lyzed has been ribonucleic acid (RNA).

Since, in the interphasic or resting nucleus, the nucleolus is considered to b
the site of RNA,[2, 3, 4, 5] the identification of nucleoli and the observations o
their variability have been based on the cytochemical localization of RNA b
utilization of the methyl green-pyronin Y method.

The Methyl Green-Pyronin Y Method

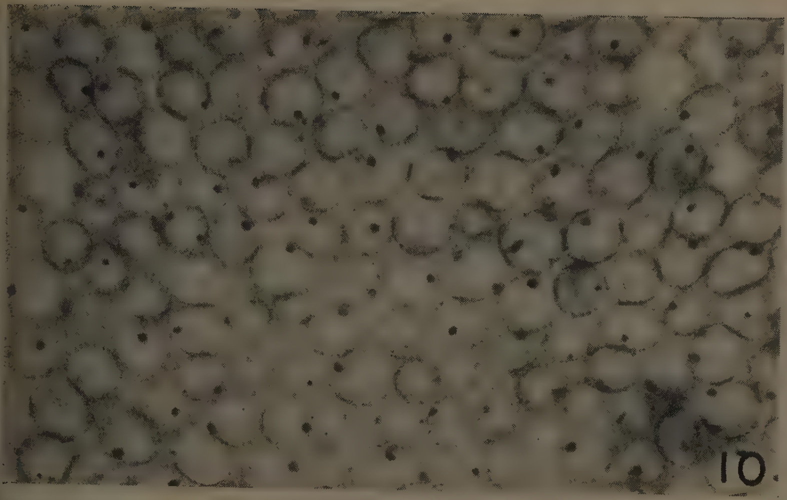
Details of the procedure for the methyl green-pyronin Y method, as use
in our laboratory, are presented in the APPENDIX to this paper. Sections fro
methyl alcohol fixed tissue and methyl alcohol fixed tissue smears are reacte
with a purified buffered methyl green-pyronin Y solution.[17] Jacobson ar
Webb,[9, 10] in a series of carefully controlled experiments (*in vitro*, enzyme dige
tion, and enzyme inhibitor methods) showed that choice of fixative and regul
tion of paraffin embedding temperature[8] were important factors in the cyt
logical preservation of nucleic acids in paraffin section methods.

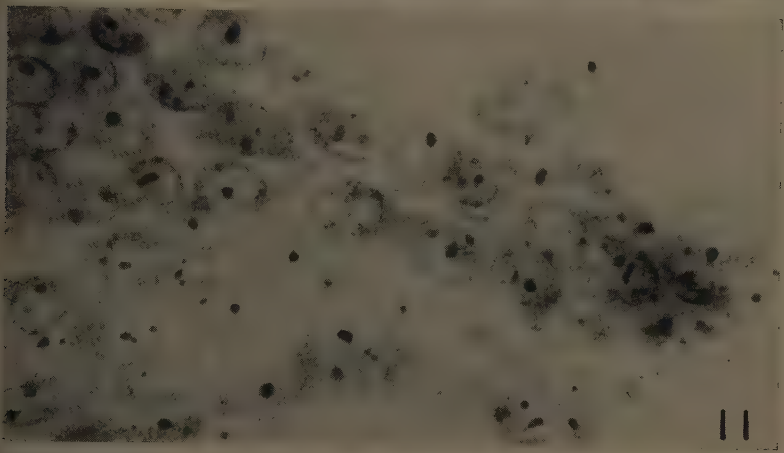
Spot checks on the specificity of pyronin Y for RNA are made from time
time by subjecting one slide to purified ribonuclease digestion and a secon
slide of the same tissue to the ribonuclease solvent. These enzyme exper
mental and control slides are reacted for one hour at room temperature, the
are reacted with the staining solution. The resulting enzyme-digested prepar
tion shows complete removal of nucleolar RNA (FIGURE 16). The rose-pi
nucleoli in the solvent control slide indicate that the solvent alone has no effe
on the preservation of RNA (FIGURE 15). Confirming Brachet and Shaver
we find that distilled water (pH 6.3 to 6.6) is as effective a solvent for the e
zyme as any of the buffer solutions used by other investigators[11, 16] and tha
when the enzyme reaction is carried out at 37° C., the solvent alone remov
the RNA pyroninophilic substance.

FIGURE 10. Ovary: papillary pseudomucinous cystadenoma. Benign tissue smear. Relatively small sing
rounded nucleoli per nucleus predominate. Some binucleolar nuclei. ×918.

FIGURE 11. Ovary: papillary pseudomucinous cystadenocarcinoma. Borderline tissue smear. Single n
cleoli larger than those in benign nuclei of FIGURE 10. Irregularly shaped nucleoli and nuclei with multip
nucleoli varying in size also present. ×918.

FIGURE 12. Ovary: papillary pseudomucinous cystadenocarcinoma. Grade II tissue smear. Single nucle
larger than in nuclei of benign and borderline cases. Note large, irregularly shaped single nucleolus in uppe
most nucleus and vacuolated nucleolus in lower left of figures. ×918.





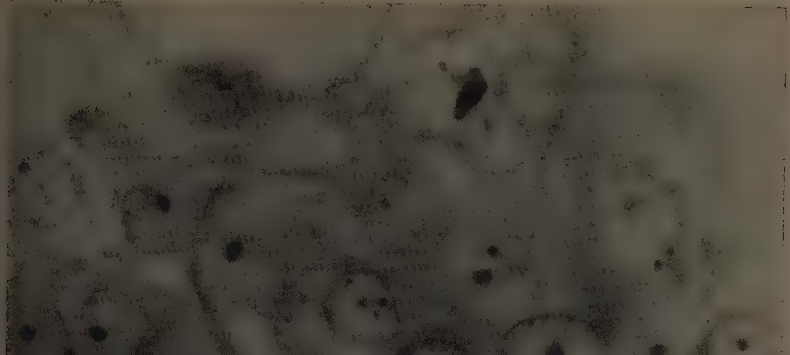
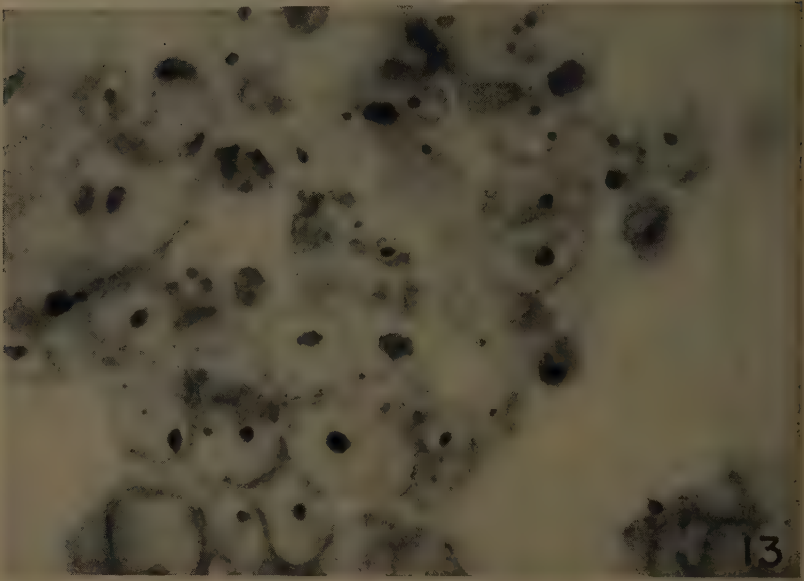


Plate III



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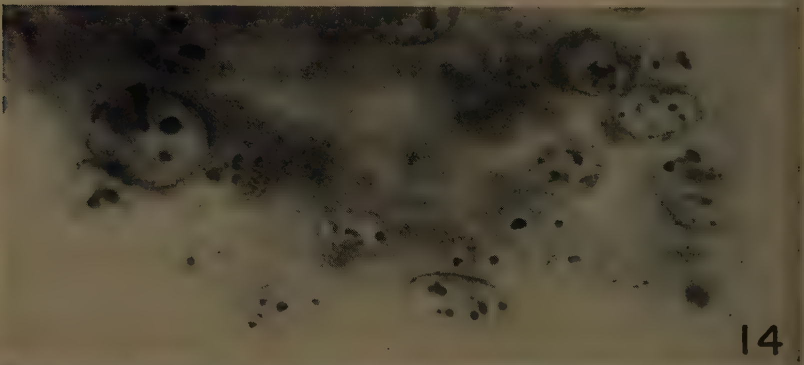

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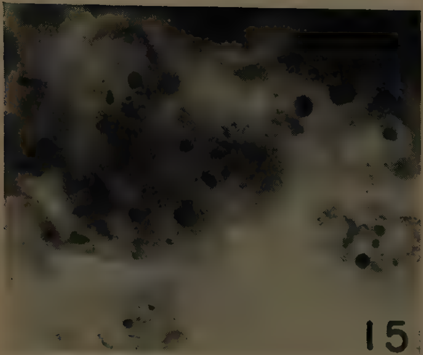

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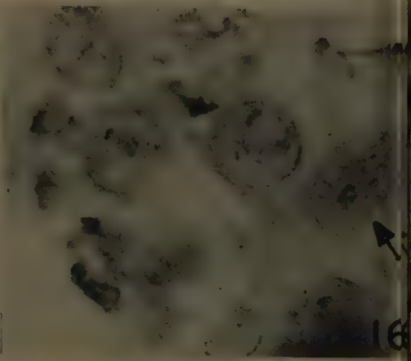

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Plate IV

This procedure has been presented in some detail in this paper because of the well-known difficulties that have plagued its use.[11, 12] The procedure as used in our laboratory has presented clear-cut and consistent results when the reaction schedule is rigidly followed. In our finished preparations, desoxyribonucleic acid (DNA) is visualized as a blue-green substance, and RNA as rose-pink. The distinctive rose-pink reaction of nucleolar RNA makes it relatively easy to distinguish the pyroninophilic nucleolar bodies in interphasic nuclei.

Nucleolar Variability in Ovarian Papillary Serous Cystadenoma and Cystadenocarcinomas

The nucleolar status in 36 histologically graded ovarian papillary serous tumors has been analyzed. In these tumor nuclei, nucleoli occur as single or multiple structures. Variations in the numbers of nucleoli per nucleus and in the diameters of single nucleoli per nucleus have been determined. Diameters of single nucleoli per nucleus have been measured with an ocular micrometer scale. Counts and measurements have been done on both sections and tissue smears. Tissue smears are advantageous for biometric studies, since they present whole nuclei for nucleolar analysis. It may be mentioned, however, that usually, for each case, the range of nucleolar numbers per nucleus and the range in single nucleolar diameters determined from the whole nuclei of tissue smears was also observed in tissue sections.

Number of nucleoli. The number of nucleoli per 200 nuclei were counted in tissue sections and tissue smears of each tumor. Benign tumor nuclei contained one to four nucleoli per nucleus (FIGURES 1 and 5). In the nuclei of cystadenocarcinomas of borderline grade, one to four nucleoli were observed (FIGURE 7). Nucleolar numbers per nucleus in cystadenocarcinomas of Grades I to III ranged from 1 to 24 (FIGURES 2, 3, 4, 6, and 8).

An analysis of the percentage relationship of numbers of nucleoli per nucleus in the five histological grades is presented in TABLE 1.

TABLE 1 clearly shows the trend of more nucleoli per nucleus with increased grades of malignancy. Although nuclei in both the benign and borderline groups may contain four nucleoli, nuclei with four nucleoli occur four times more frequently in borderline than in benign tumors. The nuclear populations of Grades I to III always demonstrated some nuclei with more than five nucleoli per nucleus. Grade III nuclear populations, however, show a significantly higher percentage of such nuclei than those of Grade I.

It is well known, from cytological evidence,[1, 6, 7] that normally, during the mammalian mitotic cycle at late anaphase or during telophase, a specific num-

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FIGURE 13. Uterus: endometrial adenocarcinoma. Grade III tissue smear. Large and multiple nucleoli per nucleus, varying in size. Such nucleolar variability does not occur in normal postmenopausal endometrium. ×918.

FIGURE 14. Cervix: epidermoid carcinoma. Grade II tissue smear. Large and multiple nucleoli, varying n size. ×918.

FIGURES 15 and 16. Control and ribonuclease tests on tissue smears of case of epidermoid carcinoma of the ervix. FIGURE 16 smear reacted in ribonuclease solution, FIGURE 15 smear in enzyme solvent, both smears then eacted with methyl green-pyronin Y solution. Solvent alone does not remove pyroninophilic nucleolar RNA (FIGURE 15). Enzyme removes nucleolar RNA so that nucleolar bodies do not react with pyronin Y (FIGURE 6). Arrow indicates nucleolar body negative for RNA but with its frequently seen DNA rim.

TABLE 1
OVARIAN PAPILLARY SEROUS TUMORS
36 CASES: % OF NUCLEOLI PER NUCLEUS

Histological grade	No. of nucleoli per nucleus			
	2	3	4	5+*
Benign	86.0	12.5	1.5	
Borderline	67.5	26.5	6.0	
Grade I	52.0	32.5	12.0	3.5
Grade II	41.0	27.0	19.0	13.0
Grade III	27.5	20.5	16.0	36.0

*Nuclei with more than 5 nucleoli per nucleus are reported in the 5+ column.

ber of nucleolar organizer chromosomes produce a specific number of nucle that generally fuse into a single nucleolus, visible as such in the interphasic resting nucleus. Since, as indicated in TABLE 1, increased numbers of nucle per nucleus are encountered in tumors of increased grades of malignancy, it suggested that, with increased grade of malignancy, there is an increased nu ber of nucleoli organized per nucleus that may show a decreased tenden towards fusion.

Size of nucleoli. Multiple nucleoli per nucleus frequently show variations size (FIGURES 2, 3, 4, and 6). Such variations in size of multiple nucleoli a more characteristic of the less differentiated tumors (Grades II and III).

Single nucleoli per nucleus also occur among the nucleolar populations tumor nuclei. Such single nucleoli are larger in the nuclei of Grade III tum (FIGURES 2, 3, 6, and 9) than they are in nuclei of benign tumors (FIGURES and 5). TABLE 2 presents the mean nucleolar diameters of single nucleoli nucleus in the five histological grades under consideration.

It is shown in TABLE 2, as indicated by measurements of nucleolar diamete that the higher the grade of malignancy evidenced by a tumor, the larger w the single nucleoli per nucleus. Single nucleoli in Grade III tumors were most three times as large as single nucleoli in nuclei of benign tumors.

In TABLE 2, the mean nucleolar diameter measurements represent the me

TABLE 2
OVARIAN PAPILLARY SEROUS TUMORS
36 CASES: MEAN NUCLEOLAR DIAMETER IN NUCLEI WITH 1 NUCLEOLUS

Histological grade	Mean in mm.	SD*	SE_m†
Benign	.00131	.00011	.00005
Borderline	.00154	.00002	.00001
Grade I	.00149	.00025	.00011
Grade II	.00200	.00054	.00017
Grade III	.00297	.00043	.00015

* SD, standard deviation of mean = $\sqrt{\frac{\Sigma d^2}{N}}$ where d is the difference from the mean, N the numbe observations.

† SE_m, standard error of mean = $\frac{SD}{\sqrt{N}}$.

TABLE 3
STATISTICAL COMPARISONS BETWEEN HISTOLOGICAL GRADES
MEAN NUCLEOLAR DIAMETERS IN NUCLEI WITH 1 NUCLEOLUS

Comparisons	Difference between means	SE diff.*	Critical† ratio	Probability
Benign vs. Borderline	+0.00023	0.00005	4.5	<0.001
Borderline vs. Grade I	−0.00005	0.00011	0.5	0.62
Grade I vs. Grade II	+0.00051	0.000202	2.5	0.012
Grade II vs. Grade III	+0.00097	0.000227	4.3	<0.001
Benign vs. Grade III	+0.00166	0.000158	10.5	≪0.001

* $SE_{diff.}$, standard error of difference between means = $\sqrt{SE_{m_1}^2 + SE_{m_2}^2}$ where SE_{m_1} and SE_{m_2} are Stand-
rd Error of the respective means.
† Critical ratio = difference between means/$SE_{diff.}$.

of samples of the nucleolar population for each grade. The small SD and SE_m calculations for each group indicate that, within each group population, the variation from the mean diameters of single nucleoli is quite small.

TABLE 3 is presented to show the derivation of the critical ratio between groups and thereby the probability of the difference between the means of two groups occurring by chance.

Probability of less than 1 per cent indicates significance, whereas probability greater than 5 per cent denotes lack of significance; between 1 per cent and 5 per cent, probability of chance occurrence is doubtful. It is shown in TABLE 3, therefore, that the chance occurrence of the difference between the mean values of nucleolar diameters in nuclei with one nucleus for benign and border-ine, Grade II and Grade III, and benign and Grade III is quite remote. In addition, it is seen that the difference between the means of nucleolar diameters of borderline and Grade I tumors is not significant. The difference between the means in Grades I and II is in the dubious class, and may prove significant with further study.

Other variability features that occur among the nucleolar populations of ovarian papillary cystadenocarcinomas are irregularity of nucleolar shape (FIGURES 2, 6, and 8) and nucleolar vacuolization (FIGURE 9). These nucleolar modifications may be explained by incomplete and erratic fusion of multiple nucleoli.

Nucleolar Variability in Other Gynecological Tumors

Similar nucleolar variations have been encountered among the nucleolar populations of the ovarian pseudomucinous (FIGURES 10, 11, and 12), the ndometrial (FIGURE 13), and the cervical epidermoid tumors (FIGURE 14). A ufficient number of these tumor types have not accumulated as yet in the project series to permit the type of biometric study that has been presented or the ovarian serous group.

Following radiation in epidermoid carcinoma of the cervix, there appears to be an enhancement of the nucleolar variations that occur in untreated malig-nant nuclei of the same case, if the primary tumor is radiation sensitive. FIGURE 4 in the paper "Studies on Nucleoprotein Patterns in Radiosensitivity Testing" by Gusberg, Tovell, Long, and Hill[19] presented in this volume shows nucleolar changes following radium therapy. The illustration on the left this figure represents the nucleolar condition in the untreated cervical lesio relatively large, multiple nucleoli, varying in size. Two weeks after the initi tion of radium treatment, as shown in the illustration on the right, the nucle were larger and more vacuolated than before treatment.

Summary and Analysis

This paper presents an analysis of nucleolar variability in gynecologic tumors in relation to their several degrees of malignancy. The types of tum include ovarian papillary serous and pseudomucinous cysts, endometri carcinomas, and cervical epidermoid carcinomas (preradiotherapy and pos radiotherapy). Nucleoli were visualized in these tumor nuclei by utilizati of the methyl green-pyronin Y method.

With increased grade of malignancy, we find, in the tumors so far examine the following nucleolar variations: (1) multiple nucleoli; (2) larger size of sing nucleoli; (3) variations in size of multiple nucleoli; and (4) irregularly shap and vacuolated nucleoli.

Presumably, these nucleolar variations, which are more apparent bo quantitatively and qualitatively in tumors of the higher grades of malignan may be explained on a cytological basis by effects of the mechanisms of pol ploidization, as described by Doctor Hsu[20] elsewhere in this monograph.

In ovarian papillary serous tumors, nucleolar counts per nucleus indica that the nuclei of benign tumors contain one to four nucleoli per nucle With increased grade of malignancy, the nuclei contain more nucleoli, Gra III nucleolar numbers ranging from 1 to 24. Associated with increased nu bers of nucleoli per nucleus is increased size of single nucleoli per nucleus.

Statistical comparisons between histological grades of the serous tumo were shown for the mean nucleolar diameters of single nucleoli per nucleus. would seem, from this type of analysis, that the chance occurrence of the diff ence between the mean nucleolar diameters for benign and borderline, Grade II and Grade III and benign and Grade III is quite remote.

In his usual approach to the tumor problem, the pathologist looks on canc tissue as an entity, and on a tumor as either benign or malignant. Followi this general analysis of tumor tissue, then, when we examine the nucleoli o tumor type such as the ovarian papillary serous neoplasms as a whole, c siderable nucleolar variability is encountered. The project of which this port is a part considers malignancy not as an entity but as a quality possess to a different degree by specific tumors that manifest different degrees or gra of malignancy. When analysis of the nucleolar population for each grade considered biometrically, it would seen, so far as the present study has p gressed, that, within each grade, nucleolar constancy exists.

Variations in such criteria as have been reported here, between tumors different grades of malignancy, may be as significant as differences betwe benign and malignant, and the study of these differences may thus serve a new approach to a more definitive diagnosis between benign and maligna neoplasms.

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APPENDIX

Methyl Green-Pyronin Y Method

I. Paraffin method

(1) Fixation: Absolute methyl alcohol . . . . 24 hour.
Change once soon after fixation.
Small pieces of tissue 2 to 3 mm. thick.
Fibrous tissue cuts with difficulty.

(2) Dehydration: Absolute n-butyl alcohol . . . . ½ hour.
Absolute n-butyl alcohol change . . . . ½ hour.

(3) Embedding: Alcohol-paraffin mixture in oven at 56° C . . . . 1 hour.
Mixture: ½ n-butyl alcohol + ½ melted paraffin.
Oven temperature must not exceed 56° at any time.
Pure paraffin, four changes during . . . . 2 hours.
Embed.

II. Tissue smear method

(1) Making smears: Tease small piece of tissue on slide.
Moisten tissue with physiological saline solution.
Distribute in thin film.
Do not allow film of cells to dry.

(2) Fixation: Place slide face down carefully in shallow dish of Absolute methyl alcohol, supporting ends of slide with glass rods. Immediately can be transferred to methyl alcohol in Coplin jar (screw top).
Fix . . . . several to 24 hours.
Slides can remain in methyl alcohol indefinitely.

II. Ascites fluid smear method

(1) Shake bottle of fluid thoroughly so that sediment is well distributed throughout fluid.
(2) Centrifuge samples of fluid at 3000 + RPM—5 min.
(3) Decant supernatant fluid from centrifuge tube.
(4) With straight tube dropping pipette (6″) transfer sediment from centrifuge tube to glass slide. Distribute in thin film.
(5) Fix fluid film as in tissue smear method.
Do not allow film to dry.

IV. Preparation of methyl green-pyronin Y staining solution.

(1) Acetate buffer solution (0.2 M pH 4.2).
  Use analytical reagents.
    Glacial acetic acid M.W. 60.05.
    Sodium acetate M.W. 136.09.
  For 0.2 M acetic acid—1.15 cc. acetic acid + 98.85 cc. dis. water.
  For 0.2 M sodium acetate—2.72 g. sodium acetate in 100 cc. dis. water.
  Mix 75 cc. of 0.2 M acetic acid + 25 cc. 0.2 M sodium acetate for pH 4.16. A
    just pH with sodium hydroxide or acetic acid.
(2) Purification of methyl green in acetate buffer.
  Wash 0.5% methyl green in acetate buffer with chloroform in a separatory funn
    until all violet color has been removed from the stain-buffer solution. Let so
    tion stand in open flask until residual chloroform has evaporated—approximate
    overnight.
  Use commission-certified methyl green. Different batches of methyl green appe
    to present varying shades of blue-green in the final reaction result with DNA.
(3) Purified methyl green-pyronin Y solution.
  100.0 cc. purified methyl green in pH 4.2 acetate buffer.
    0.1 g. pyronin Y (commission-certified).
  Keep refrigerated in glass-stoppered bottle.
  Solution may be reused for months.
  Bring to room temperature before using.

V. Methyl green-pyronin Y staining procedure

(1) (*a*) Sections (5 μ).
    Decerate and hydrate from absolute ethyl alcohol in fresh alcohols to two qu
      rinses in distilled water. Let stand in 50 per cent alcohol if necessary, r
      distilled water.
  (*b*) Tissue and ascites fluid smears
      directly into absolute alcohol and proceed as in (*a*).
(2) Stain: sections..*5 minut*
    smears..*1 ho*
(3) Handle each slide individually.
(4) *Quick* plunge into distilled water. Use fresh water frequently.
(5) Quick blot with No. 1 filter paper (7 cm. circles are convenient).
(6) Immediately into tertiary butyl-alcohol mixture....................½ minu
    with continuous agitati
  Alcohol mixture
    Tertiary butyl alcohol..3 par
    Absolute ethyl alcohol..1 par
(7) *Quick* rinse xylene.
(8) Xylene, two changes, five minutes each.
  Wipe forceps dry before carrying another slide through procedure from stain
    staining solution will not be contaminated by xylene.
(9) Mount slide in xylene-balsam media.

VI. Ribonuclease test

(1) Hydrate 2 (*a* and *b*) slides of same case to distilled water as in V 1. (*a*) and (*b*). .
(2) (*a*) Enzyme reaction
    0.01 per cent ribonuclease in distilled water, room temperature for 1 hour.
    Purified ribonuclease obtained from Worthington Biochemical Corporati
    Freehold, N. J.
  (*b*) Solvent control
    Distilled water, room temperature for 1 hour.
(3) Stain slides *a* and *b* at same time as in section V (2)–(9).

INTRAEPITHELIAL CARCINOMA OF THE UTERINE CERVIX: A HISTOCHEMICAL AND CYTOMORPHOLOGICAL APPROACH*

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Additional information concerning the currently doubtful relationship of intraepithelial carcinoma to invasive squamous carcinoma of the uterine cervix was sought through the following investigations:

(1) Histochemical localization of protein-bound sulfhydryl and disulfide groups. Sulfhydryl groups are important in various types of cellular metabolism. The formation of disulfide groups is intimately related to keratinization.

(2) Histochemical localization of sites of dehydrogenase activity. This enzyme is important in cell respiration.

(3) Quantitation of nuclear size and nuclear-cytoplasmic ratio. These are widely discussed with respect to the histological diagnosis of intraepithelial carcinoma.

(4) Photometric estimation of hyperchromatism. This is another cell characteristic closely evaluated in the diagnosis of intraepithelial carcinoma.

Each of these studies reviews a property of the cell concerned with its growth or function. They should assist in a more accurate delineation of the relationship of intraepithelial to invasive squamous carcinoma of the uterine cervix.

Protein-bound sulfhydryl and disulfide groups having relationship to growth and maturation of squamous epithelial cell have been studied in 87 biopsies of the uterine cervix.[1] Protein-bound sulfhydryl groups are essential for the activity of many enzymes.[2] They serve as protein links and are important in cell division and growth.[3] The presence of sulfhydryl groups in the epidermis is closely related to the process of keratinization,[4] in which they undergo a transformation to disulfide substances in the stratum corneum. Previously we[5] found sulhydryl groups in the basal and malpighian layers of normal epidermis and in the neoplastic cells of basal and squamous cell carcinoma. Disulfide groups were found in keratinizing cells of normal epidermis and in keratinizing cells and epithelial pearls where present in skin carcinoma.

Dehydrogenase enzymes play a vital role in the respiratory processes of many living cells. They form a link in the chain of reactions concerned with oxidation of lipids, carbohydrates, and proteins.[6] Tetrazolium salts as indicators of dehydrogenase activity in living cells are recent additions to the histochemical armamentarium. Neotetrazolium (NT) is reduced to a formazan in the presence of dehydrogenase enzymes.[8] In the presence of an excess of succinate, much of the reaction is related to the presence in the cells of succinic dehydrogenase.[8, 9] Dehydrogenase enzyme distribution was found to corre-

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* This investigation was aided by Research Grants C-1486, C-1711, C-2111 and C-2112 from the National Cancer Institute, National Institutes of Health, United States Public Health Service, Bethesda, Md. Portions of these studies were done in the departments of Pathology, Emory University School of Medicine, Atlanta, Ga., and the University of Texas, M. D. Anderson Hospital and Tumor Institute, Houston, Texas.

spond with regions of cell growth in cervical biopsies from pregnant and nonpregnant women.[10] In other investigations, dehydrogenase activity was found in the basal layer of normally maturing squamous epithelium and, more diffusely, through metaplastic epithelium, intraepithelial carcinoma, and invasive squamous carcinoma of the cervix.[11, 12]

The next phase of the study was an attempt to quantitate nuclear size and nuclear:cytoplasmic ratio as histologic criteria in the diagnosis of carcinoma of the uterine cervix.[13] It was believed that, if these measurable facets of tissue diagnosis could be expressed in terms of number, more ready comparison of histologic diagnoses in different laboratories would be facilitated. We hoped to establish a set of measurement properties that would apply to frank invasive carcinoma, and a set of measurement properties from nonneoplastic conditions of the cervix such as squamous metaplasia. Then, by applying the same measurement methods to intraepithelial carcinoma, we hoped to learn in which measurements it conformed exactly to invasive carcinoma, and in which i failed to exhibit nuclear measurement properties of undoubted invasive carcinoma.

The fourth phase of the study concerned estimation of hyperchromatism as manifested by light absorption employing photometric methods and using:[1] (1) the type of section usually available to the average pathologist; (2) ligh in the visual range; and (3) simple photometric apparatus.[15]

Intraepithelial carcinoma conformed closely to invasive carcinoma in all four of these chemical and morphological approaches, each relating in some manner to cell growth.

Materials and Methods

In the sulfhydryl and disulfide study, biopsies from 27 cases of invasive squamous cell carcinoma, 3 cases of intraepithelial carcinoma and 57 cases of nonneoplastic cervical lesions were studied. For sulfhydryl localization, th technique of Barrnett and Seligman[2] was employed. Disulfide was detected by treating sections with 0.1 M sodium iodoacetate to block pre-existing sulfhydry groups and, after washing, by next immersing the slides in a solution containing, per 100 ml., 10 gm. sodium acetate and 5 gm. sodium sulfite, and finally after washing, by carrying out the sulfhydryl reaction. Sections from all blocks were stained with hematoxylin and eosin for comparison purposes.

In the dehydrogenase study, cervical tissue from 37 women having primaril noncervical disease and 15 women having intraepithelial or invasive squamou cell carcinoma were incubated in neotetrazolium (1 per cent) with sodium succinate (0.03 M). Frozen sections were cut. Hematoxylin and eosin sections were made for comparison.[12]

In the nucleus measurement study,[13] standardized photomicrographs from normal and abnormal epithelium of 25 cases each of invasive carcinoma, intraepithelial carcinoma and squamous metaplasia of the cervix were prepared. They were projected at a total magnification of 2300, and the outlines of nuclei in sample areas of basal layer, superficial layer, and middle layer wer traced on graph paper. These were measured with a planimeter. The mea values of percentage area occupied by nuclei and of average area of whole nuclei in normal and abnormal epithelium of the 25 cases were obtained and subjected to analysis.

In the photometric study,[14] normal and abnormal epithelium (as seen in hematoxylin and eosin sections) from 20 cases each of invasive squamous cell carcinoma and squamous metaplasia of the cervix were examined with a fluorescent light source and an electronic photometer.[15] Readings were taken with no filter and with a Wratten red 25 A filter.

The normal epithelium in both photometric and nucleus measurement studies was used as a control of tissue preparation technique.

Results

In the sulfhydryl preparations,[1] the specific reaction was indicated by red-to-blue staining of cytoplasm and nucleoli.[2] Similar staining of pertinent areas was found in the sections prepared to show disulfide groups only. In portio vaginalis epithelium, considerable sulfhydryl group reaction was found in cells of the basal and immediately superjacent layers. More superficial layers showed a much weaker reaction in general. Disulfide reaction was found only in the superficial cell layers, the strength of the reaction degree of keratinization. In metaplastic epithelium, evidence of sulfhydryl reaction was more uniform through the cell layers. Only slight evidence of disulfide reaction was found, and this in the most superficial cell layers. Intraepithelial carcinoma showed evidence of sulfhydryl reaction in all of the neoplastic cells. Disulfide reaction was present minimally in the superficial layers. Invasive carcinoma without material keratinization or epithelial peral formation revealed sulfhydryl reaction in the neoplastic cells and little or no disulfide reaction. When keratinization was more prominent, the epithelial pearls and keratinized cells contained a moderate sulfhydryl reaction and a considerable disulfide reaction. The disulfide reaction of the keratinized cells was striking in comparison with the paucity of staining of adjacent neoplastic cells.

Evidence of dehydrogenase activity was largely confined to the basal layer of normally maturing portio vaginalis epithelium.[12] In metaplastic epithelium, dehydrogenase reaction was maximal in the basal layer but extended diffusely through the epithelium. In general, the diffuseness of formazan deposition indicating dehydrogenase activity varied inversely with the degree of maturation that the cells manifested. In specimens of intraepithelial carcinoma, heavy evidence of dehydrogenase activity extended diffusely through the epithelial layer. Enzyme activity was usually still somewhat more pronounced in the basal layer. Invasive squamous cell carcinoma also showed evidence of dehydrogenase activity in all viable cells. No enzyme activity was seen in epithelial pearls, however, and little was found in adjacent keratinizing squamous cells. In no instance did neoplastic epithelium show greater evidence of dehydrogenase activity than that seen in the basal layer of normal portio vaginalis epithelium.

Data relating to mean nuclear area in the epithelial samples of the nucleus measurement study[13] are summarized in TABLE 1. Normal epithelium from

TABLE 1

MEAN NUCLEAR AREA FROM TWENTY-FIVE CASES EACH OF INVASIVE SQUAMOUS CELL CARCINOMA, INTRAEPITHELIAL CARCINOMA AND SQUAMOUS METAPLASIA OF THE CERVIX

	Mean nuclear area (square microns) (and standard errors)			IV (I vs. II)		V (I vs. III)		VI (II vs. III)	
	I Invasive carcinoma	II Intraepithelial carcinoma	III Squamous metaplasia	"t"*	"p"	"t"	"p"	"t"	"p"
Normal									
a. Basal layer	32.91 ± 2.68	43.87 ± 3.66	36.56 ± 3.17	2.419	$0.02 > p > 0.01$	0.880	$0.4 > p > 0.3$	1.150	$0.2 > p > 0.1$
b. Middle layer	30.47 ± 1.95	35.35 ± 3.17	30.47 ± 1.95	1.311	$0.2 > p > 0.1$	0.000	$p \simeq 1.0$	1.311	$0.2 > p > 0.1$
c. Superficial layer	18.28 ± 0.80	20.72 ± 1.71	19.50 ± 1.22	1.292	$0.3 > p > 0.2$	0.8336	$0.5 > p > 0.4$	0.581	$0.6 > p > 0.5$
Abnormal									
d. Basal layer	46.31 ± 4.14	54.85 ± 7.37	39.00 ± 3.17	1.010	$0.4 > p > 0.3$	1.402	$0.2 > p > 0.1$	1.976	$0.1 > p > 0.05$
e. Middle layer	53.63 ± 4.39	57.28 ± 4.38	45.10 ± 3.41	0.589	$0.6 > p > 0.5$	1.535	$0.2 > p > 0.1$	2.194	$0.05 > p > 0.02$
f. Superficial layer	45.10 ± 3.17	46.31 ± 3.66	31.69 ± 2.07	0.250	$0.9 > p > 0.8$	3.541	$0.01 > p > 0.001$	3.478	$0.01 > p > 0.001$

* The symbol "t" represents the significance ratio; "p" is the probability of a greater value of "t." For example, $0.8 > p > 0.7$ means that there is 70 to 80 per cent chance that the difference under analysis is mere coincidence.[17,18]

cases of invasive carcinoma, intraepithelial carcinoma, and squamous metaplasia showed no differentiating features. Measurement values in intraepithelial and invasive carcinoma did not differ strikingly. Both types of carcinoma had greater mean nuclear size in middle and superficial layers than did metaplastic epithelium.

Data relating to percentage of area occupied by nuclei (one way of expressing nuclear:cytoplasmic ratio) in the various epithelial samples are summarized in TABLE 2. Again normal epithelium from all three types of cases had no differentiating features. Intraepithelial and invasive carcinoma had similar measurement properties, markedly larger than those of metaplastic epithelium.

Data relating to the photometric measurements are summarized in TABLE 3. These determinations showed no differentiating features of normal epithelium from all three types of cases. Neoplastic epithelium from invasive carcinoma and intraepithelial carcinoma had similar mean optical densities, these being markedly higher than that of metaplastic epithelium, whether or not the red filter was used.

Discussion

In general, viable functioning cells showed evidence of sulfhydryl groups. Disulfide groups were present in keratinizing cells. Sulfhydryl groups are important in varied cell functions: in the coagulation of blood, in cell permeability, and in hormone synthesis and activity.[3] The role of sulfhydryl groups in the growth of tumor tissue is said to be a corollary of the function of these groups in cell division and growth.[3] They are very sensitive to ionizing radiation, being diminished by small doses of X radiation. The distribution of sulfhydryl and disulfide groups offers no findings specific for either intraepithelial or invasive carcinoma. Evidence of sulfhydryl groups was found in all of the epithelial cells, the degree of the staining reaction being almost identical in proliferating cells in the deeper layers of portio vaginalis and metaplastic epithelium and in intraepithelial and invasive carcinoma. All contained growing proliferating cells, and all contained sulfhydryl groups. Disulfide groups in this study were related to keratinization, whether occurring in neoplastic or nonneoplastic epithelium.

Sites of dehydrogenase activity corresponded generally with regions of cell growth or of active cell function such as mucus secretion in the endocervix. The technique offers no findings specific for either intraepithelial or invasive squamous cell carcinoma. The degree of formazan deposition manifesting dehydrogenase activity was almost identical in proliferating cells in the basal ayer of portio vaginalis and metaplastic epithelium and in intraepithelial nd invasive carcinoma. All contained proliferating cells and all manifested dehydrogenase activity.

The nuclear measurement study indicated that significant differences in nuclear size and nuclear:cytoplasmic ratio (as expressed in percentage of tissue occupied by nuclei) do exist in normal and pathological squamous epithelium. nvasive squamous cell carcinoma gave measurement properties generally orresponding to those of intraepithelial carcinoma. Both types of neoplastic

TABLE 2

PERCENTAGE OF AREA OCCUPIED BY NUCLEI IN NORMAL AND ABNORMAL EPITHELIUM FROM TWENTY-FIVE CASES EACH OF INVASIVE SQUAMOUS CELL CARCINOMA, INTRAEPITHELIAL CARCINOMA AND SQUAMOUS METAPLASIA OF THE CERVIX

	Mean percentage of area occupied by nuclei (with standard errors)			IV (I vs. II)		V (I vs. III)		VI (II vs. III)	
	I Invasive carcinoma	II Intraepithelial carcinoma	III Squamous metaplasia	"t"	"p"	"t"	"p"	"t"	"p"
Normal									
a. Basal	39.28 ± 1.97	46.30 ± 1.97	42.01 ± 1.95	2.520	$0.02 > p > 0.01$	0.986	$0.4 > p > 0.3$	1.548	$0.2 > p > 0.1$
b. Middle	10.77 ± 1.09	10.11 ± 1.12	8.77 ± 0.98	0.422	$0.7 > p > 0.6$	1.364	$0.2 > p > 0.1$	0.901	$0.4 > p > 0.3$
c. Superficial	5.05 ± 0.60	4.03 ± 0.41	3.89 ± 0.34	1.403	$0.2 > p > 0.1$	1.682	$0.2 > p > 0.1$	0.264	$0.8 > p > 0.7$
Abnormal									
d. Basal	55.72 ± 2.38	59.03 ± 2.03	47.54 ± 1.98	1.058	$0.3 > p > 0.2$	2.642	$0.02 > p > 0.01$	4.051	$p < 0.001$
e. Middle	48.03 ± 2.11	46.33 ± 2.00	28.78 ± 1.98	0.584	$0.6 > p > 0.5$	6.651	$p < 0.001$	6.246	$p < 0.001$
f. Superficial	44.46 ± 2.69	36.12 ± 2.61	21.06 ± 2.03	2.225	$0.05 > p > 0.02$	6.944	$p < 0.001$	4.554	$p < 0.001$

TABLE 3

OPTICAL DENSITIES AND RELATED DATA FROM TWENTY CASES EACH OF INVASIVE CARCINOMA, INTRAEPITHELIAL CARCINOMA AND SQUAMOUS METAPLASIA OF THE CERVIX, WITH OR WITHOUT RED (WRATTEN 25-A) FILTER, USING FLUORESCENT LIGHT

	Mean optical densities (and standard errors)			4 (1 vs. 2)		5 (1 vs. 3)		6 (2 vs. 3)	
	I Invasive carcinoma	II Intraepithelial carcinoma	III Squamous metaplasia	"t"	"p"	"t"	"p"	"t"	"p"
Normal epithelium									
a. No filter	0.254 ± 0.018	0.262 ± 0.017	0.262 ± 0.014	0.323	$0.8 > p > 0.7$	0.351	$0.8 > p > 0.7$	0	$p = 1.0$ (approx.)
b. Red filter	0.169 ± 0.012	0.182 ± 0.016	0.149 ± 0.011	0.650	$0.6 > p > 0.5$	1.227	$0.3 > p > 0.2$	1.701	$0.1 > p > 0.05$
Abnormal epithelium									
c. No filter	0.400 ± 0.021	0.415 ± 0.019	0.286 ± 0.015	0.530	$0.6 > p > 0.5$	4.419	$p < 0.001$	5.331	$p < 0.001$
d. Red filter	0.351 ± 0.025	0.356 ± 0.020	0.174 ± 0.010	0.190	$0.9 > p > 0.8$	6.580	$p < 0.001$	8.125	$p < 0.001$
Abnormal epithelium compared with normal epithelium									
e. No filter	0.158 ± 0.014	0.150 ± 0.011	0.027 ± 0.007	0.449	$0.7 > p > 0.6$	8.344	$p < 0.001$	9.462	$p < 0.001$
f. Red filter	0.186 ± 0.014	0.171 ± 0.013	0.025 ± 0.006	0.785	$0.5 > p > 0.4$	10.592	$p < 0.001$	10.210	$p < 0.001$

epithelium differed in some nuclear measurement properties from metaplas epithelium.

In recent months, a series of cases of intraepithelial carcinoma, atypic hyperplasia, and squamous metaplasia of the cervix loaned to us by Doct James Reagan have been subjected to similar analysis. This material provid us with an opportunity to apply these nuclear measurement methods to t extremely interesting entity of atypical hyperplasia. The morphology atypical hyperplasia in this group of cases has been described.[19] A furth point of interest is the fact that the diagnoses were established by Doct Reagan and the analyses done by us, thus reducing the subjective eleme somewhat. Our statistical studies are not yet complete. Preliminary revie however, suggests that atypical hyperplasia will reveal nuclear measureme properties about halfway between squamous metaplasia and intraepithel carcinoma of the uterine cervix.

The photometric studies showed: (1) virtually identical light absorpti properties, presumably correlating with intensity of staining (and this p sumably dependent on nucleoprotein content) of neoplastic epithelia from i vasive and intraepithelial carcinoma; (2) higher mean optical density of bo types of neoplastic epithelium as compared with normal or metaplastic e thelium; (3) accentuation of these differences on comparing the light-abso tion properties of normal and abnormal epithelium in individual cases.

It should be emphasized that although intraepithelial and invasive carcinom showed closely conforming properties during these studies, the methods e ployed offer little immediate diagnostic assistance. The cell properties studi (sulfhydryl group reactivity, dehydrogenase activity, nuclear:cytoplasm relationships, hyperchromatism) are, in general, related to growth propensit of the cells, and not specifically to neoplasia. Essentially similar histochemi reactions were seen in all growing squamous cells, in the basal layer of por vaginalis epithelium, in active metaplasia, and in intraepithelial and invasi carcinoma. In these same growing cells, the nucleus measurement a photometric studies revealed differences only of degree, with no sharp demar tion between all examples of one type of epithelium and all examples of anot type.

Summary

The exact relationship of intraepithelial carcinoma to invasive cervi carcinoma remains in some doubt. To orient intraepithelial carcinoma furt with respect to frank invasive carcinoma and to nonneoplastic cervical lesio histochemical localization of protein-bound sulfhydryl and disulfide grou dehydrogenase localization, measurement of nuclear size, and photomet estimation of hyperchromatism have been investigated. Each facet of t study involves a cell property more prominent during growth.

Sulfhydryl groups related to various phases of cell metabolism were fou in all viable squamous cells, whether occurring in portio vaginalis or metaplas epithelium, or in intraepithelial or invasive carcinoma. Disulfide groups w found in keratinizing surface squamous cells and in epithelial pearls a keratinizing neoplastic cells.

In normal portio vaginalis epithelium, dehydrogenase activity is largely limited to the basal layer. Evidence of this enzyme is maximal in the basal layer of metaplastic epithelium, but occurs lightly through the epithelium. Dehydrogenase activity occurred diffusely through the layers of intraepithelial and invasive carcinoma.

Intraepithelial and invasive carcinoma showed closely approximating characteristics as regards mean nuclear size and nuclear:cytoplasmic ratio.

Estimating hyperchromatism by photometric methods, intraepithelial and invasive carcinoma showed almost identical light transmission properties, using sections prepared by standard procedures. This type of comparison of hyperchromatism revealed much higher values for carcinomatous than for metaplastic or normal squamous epithelium.

Using these four methods of studying different properties of squamous epithelium, each relating in some manner to cell growth, intraepithelial carcinoma conformed closely to invasive carcinoma of the cervix as regards distribution of sulfhydryl and disulfide groups, localization of dehydrogenase activity, mean nuclear size, nuclear:cytoplasmic ratio, and hyperchromatism.

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THE ELECTRON MICROSCOPY OF NORMAL AND MALIGNANT CELLS

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Advances in the techniques of preparing tissue sections for electron microscopy have been extremely rapid since the original demonstration of the practicality of such a procedure by Pease and Baker in 1948.[1] Advances have been so rapid and continuous in fact that, until very recently, attempts at an evaluation of this approach in terms of its contributions to our knowledge in the field of cytology have been of questionable value. Now that these methods are becoming standardized, however, a preliminary inventory, of necessity still tentative, would appear to be in order.

In attempting this evaluation, we should like first to describe what is considered acceptable standard procedure for preparing tissue sections for examination with the electron microscope. Second, we should like to seek a way through the maze of new terminology while attempting to evaluate the present state of our knowledge of the ultrastructure of normal cells. Third, with the foregoing as a background, we should like to examine with you a series of neoplastic cells to determine what morphologic similarities exist and in what way, if any, they differ from normal cells.

After the original demonstration by Pease and Baker, three technical improvements have been primarily responsible for the rapid advances made in the last few years. The first of these was the introduction of *n*-butyl methacrylate as an embedding matrix by Newman, Borysko, and Swerdlow in 1949.[2] The second was the demonstration of the value of the glass knife as a cutting edge by Latta and Hartman in 1950.[3] The third was Palade's demonstration[4] of the improved quality of fixation resulting from the use of osmic acid at or near neutrality (1952).* The reduction of polymerization damage by the substitution of benzoyl peroxide for luperco (dichlorobenzoyl peroxide) as a catalyst (Howatson[6]), and rapid rather than slow dehydration through the alcohols have further improved the results obtained.

The development of new microtomes and modification of existing microtomes for ultrathin sectioning by several investigators has made it possible to cut sections routinely 20 to 50 mμ in thickness.[7-10] By using one of these microtomes and, in general, the technique outlined above, consistently good results are now being achieved in a number of different laboratories.

What immediately follows is not intended as an exhaustive review of the many contributions to our knowledge of cell ultrastructure but, rather, as a montage of a "normal" cell formed by piecing together the necessary parts by selection from specific sources.

The ultrastructure of normal cells has been occupying the attention of many nvestigators over the past several years. For various reasons, a rather bewildering assortment of terms, many applying to the same structures, has bee inserted into the literature during this interval. This has happened partl because of the newness of the field, partly because of the difficulty of relatin ultrastructure to cell components identifiable with the light microscope, an partly because of the very proper position taken by some investigators tha until this relationship is definitely established, purely descriptive terms shoul be used.

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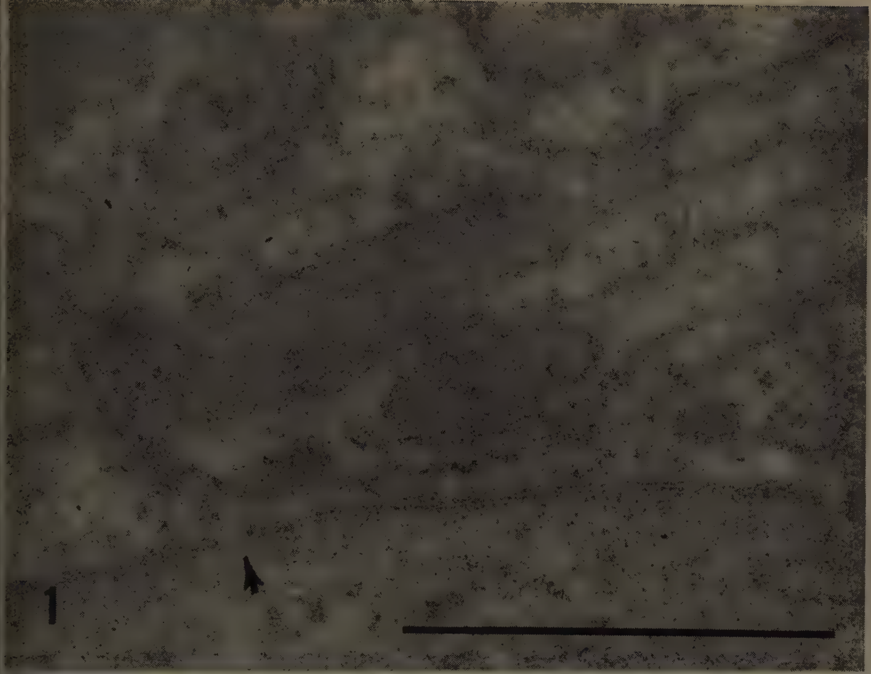
* We have recently found that a mixture of 1 per cent osmic acid, 1 per cent chrome solution ($K_2Cr_2O_7$ brought to pH 7.2 with KOH) and 0.85 per cent Na Cl serves as a useful adjunct to buffered osmic acid as a ixative.[5]

No difficulty has arisen in this regard in studies on the nucleus, probabl because discouragingly little in the way of new information concerning nuclea structure has resulted from such studies. Hartmann[11] first called attentio to the fact that the nucleus of nerve cells possesses a double membrane. I has since been determined that a double membrane is characteristic of a variet of vertebrate cell nuclei. FIGURE 1 is just another example. It illustrate the basal portion of the nucleus and some of the basal cytoplasm of a principa cell of the duodenum. Small openings in the nuclear membrane approximatel 30 mμ in diameter are visible at irregular intervals. It is very difficult to determine whether these structures are artifacts of fixation or whether they represent real openings where nucleoplasm and cytoplasm are in continuity. Th threadlike extensions from the nucleus into the cytoplasm of frog oöcytes described by Pollister[12] are less open to question. In any case, a definite answe to the question of the presence or absence of pores in the nuclear membran in interkinetic somatic cells is of considerable importance to those studyin the chemistry of the nucleus by homogenization and centrifugation technique (Hogeboom and Kuff,[13]). For this reason, an early answer to this questio is obviously desirable.

The outer of the two nuclear membranes has been called the cytoplasm membrane because of the frequent presence of cytoplasmic granules on it outer surface.[14] There is some reason to believe that this membrane is a inner cytoplasmic membrane rather than an outer nuclear membrane. Ce tainly, it reacts to late fixation much as do membranes of the cytoplasn FIGURE 2 shows the difference in the reaction of the two membranes to wha is apparently slightly delayed fixation. Several fenestrations in the membrane are also evident.

Within the nucleus, two components are distinguished: the nucleolus, o the one hand, and the remainder of the nucleoplasm on the other. The nucleolus is not an homogeneous structure. It consists basically of clusters o small granules of high electron scattering power averaging 10 to 15 mμ in diameter. These closely packed granules frequently are arranged as broa anastomosing cords forming a spongelike reticulum. Between these cords, nucleoplasmic substance of lower electron scattering power is interposed (FIGURE 3). In the absence of this latter substance, the nucleolus may appear a a solidly packed mass of the small granules. No investigators have produce evidence of the presence of a membrane or membranes associated with th nucleolus.

With the exception of the nucleolus, electron microscopy has added but litt to our knowledge of the structural organization of the interkinetic nucleu There are several possible explanations for this. One that must always b



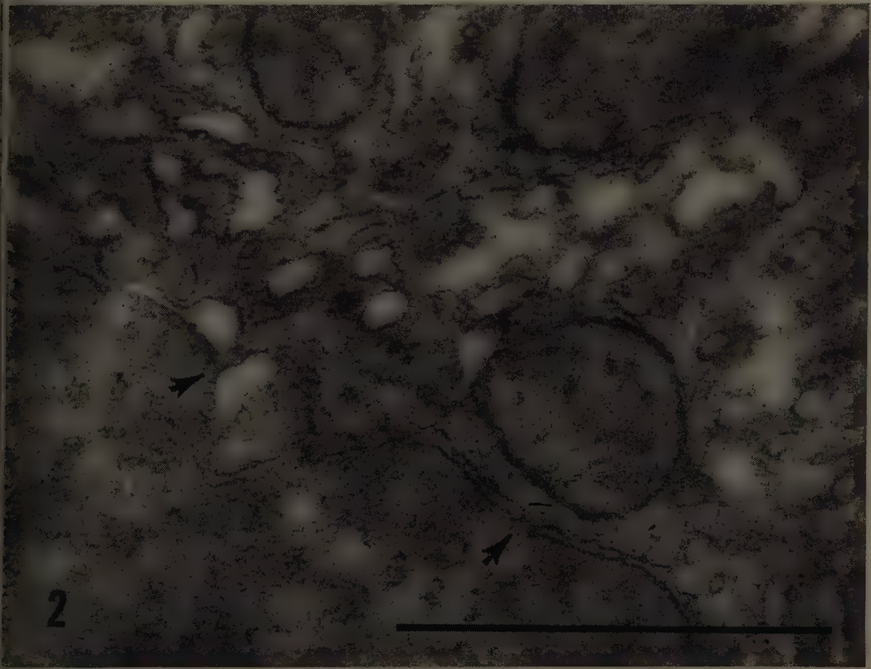


FIGURE 1. Electron micrograph of the upper portion of a nucleus and the adjoining cytoplasm of a principal cell of the duodenum of a mouse. The double nature of the nuclear membrane is apparent as is a fenestration in the membrane (arrow). Mitochondria and ergastoplasmic membranes with associated small (Palade) granules are also visible. Chrome-osmic fixation (pH 7.2). The line in this and all following figures represents 1 micron. Approximately ×57,000.

FIGURE 2. Electron micrograph of a portion of the nucleus and adjoining cytoplasm of an hepatic cell of a mouse showing evidence of slightly delayed fixation. At several points, there is continuity between cytoplasm and nucleoplasm (arrows). At one of these points, there are saclike dilations of the space between the membranes similar to those between the membranes of the adjoining ergastoplasm. Chrome-osmic fixation (pH 7.2). Approximately ×57,000.

FIGURE 3. Electron micrograph of a portion of a nucleus of a principal cell of the duodenum showing t nucleolus. Small granules comparable in size and electron scattering power to the granules of the ergastopla (FIGURE 12) are responsible for the apparently greater density of the nucleolus. Chrome-osmic fixation (pH 7. Approximately ×57,000.

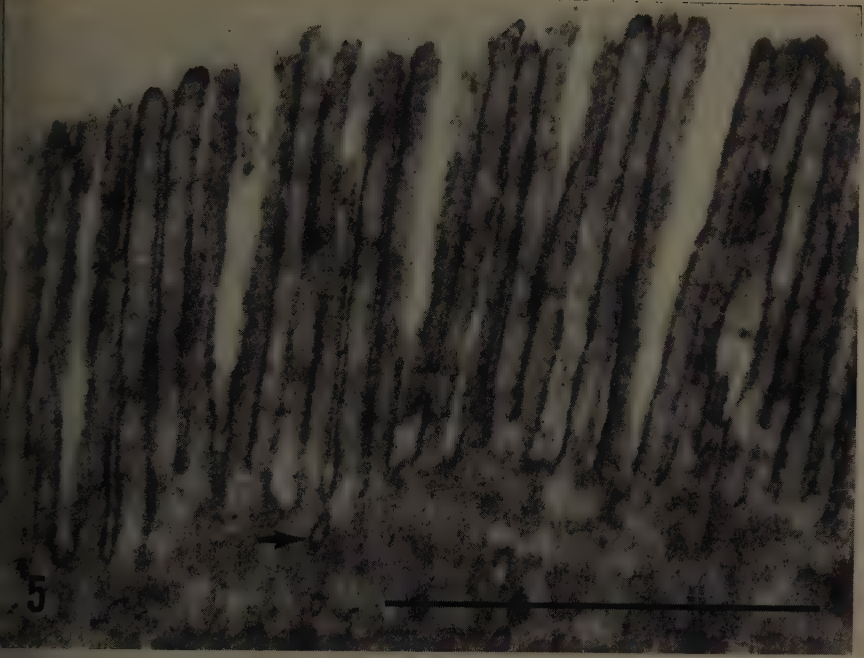
considered is the possibility that the osmic acid fixative buffered to or near neutrality may not preserve nuclear structure satisfactorily. A second is that nuclear structure is so complex as to be extremely difficult to analyze in ultrathin sections. A third is that the structural organization may be at or near the molecular level, *i.e.*, at a particle size close to, or beyond, the resolving power of the electron microscope. We suspect the actual reasons are a combination of the latter two. In the best chrome-osmium fixed preparations, organization of small granules 3 to 5 mμ in diameter, arranged in parallel rows along very thin membranes, is evident in the nucleoplasm. This organization possibly represents the details of chromosome structure in resting nuclei. (FIGURE 4)

At first glance, the ultrastructure of the cytoplasm of normal cells appears to be more complex than that of the nucleus. As suggested above, however, this relatively greater complexity may be more apparent than real. In any case, the presence of a variety of formed elements, the specialization of cell surfaces, *etc.*, made the cytoplasm a more likely area for immediately productive studies. It was found rather early that the striated border, brush border, and stereocilia, long since recognized as specializations of cell surfaces by light microscopy, consisted of regularly spaced tubular evaginations of the cell membrane. It has been suggested that such surface specializations are not remarkable, since free cell surfaces of cells in tissue culture and peritoneal fluid exhibit similar structures.[15] It is nevertheless true that the surface specializations of cells actively concerned with absorption and/or secretion are readily distinguished from the simple cell processes of free cells in tissue culture or peritoneal fluid. The former are closely and regularly spaced, of consistent length and diameter, and are not broad-based. The latter show none of these characteristics (compare FIGURES 5 and 6).

In some organs, notably the kidney and the liver, endothelial cells of certain capillaries or sinusoids exhibit fenestrated membranes. Hall[16] first described them in glomerular capillaries, and Pease[17] recently described them in capillaries associated with tubular components of the nephron. These pores or fenestrations vary somewhat in size but average 35 mμ in diameter. They may be demonstrated with a variety of fixatives and, presumably, they are not artifacts. Under these conditions, then, only the basement membrane of the capillary would separate blood plasma in glomerular capillaries from Bowman's space (FIGURE 7). A similar situation exists under certain conditions in the liver. What these conditions are is not known at present, but the cytoplasm of the lining cells of some sinusoids shows fenestrations (FIGURE 8) while that of others does not (FIGURE 9).

No particular problem in terminology has arisen in connection with the description of surface specialization of cells, nor with the fine structure of mitochondria. Mitochondria, however, do exhibit a very complex and interesting internal structure as demonstrated first by Palade in 1952[18] and, shortly thereafter and independently, by Sjöstrand.[19] Internal membranes, called by Palade "christae mitochondriales" are double and are usually continuous with the inner of the two limiting membranes of the mitochondrion. The complexity, number, spacing, and distribution of these membranes are surprisingly consistent in any one-cell type, but they vary over an extremely wide range

FIGURE 4. Electron micrograph of the nucleoplasm of a nucleus of a principal cell of the duodenum showi the alignment of small granules in association with thin membranes (arrows). Part of a nucleolus is visi at the upper left of the figure. Chrome-osmic fixation (pH 7.2). Approximately ×79,500.



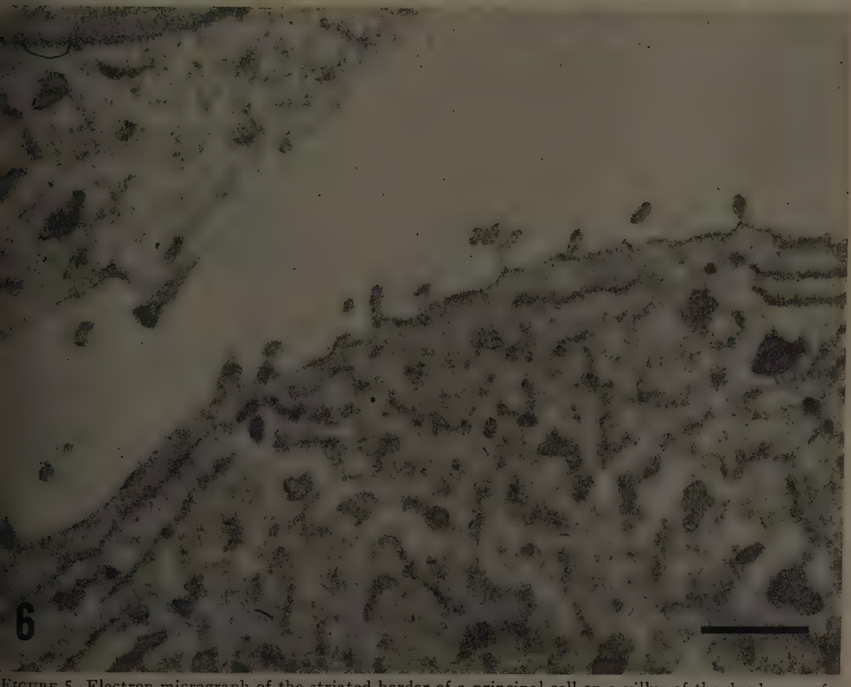
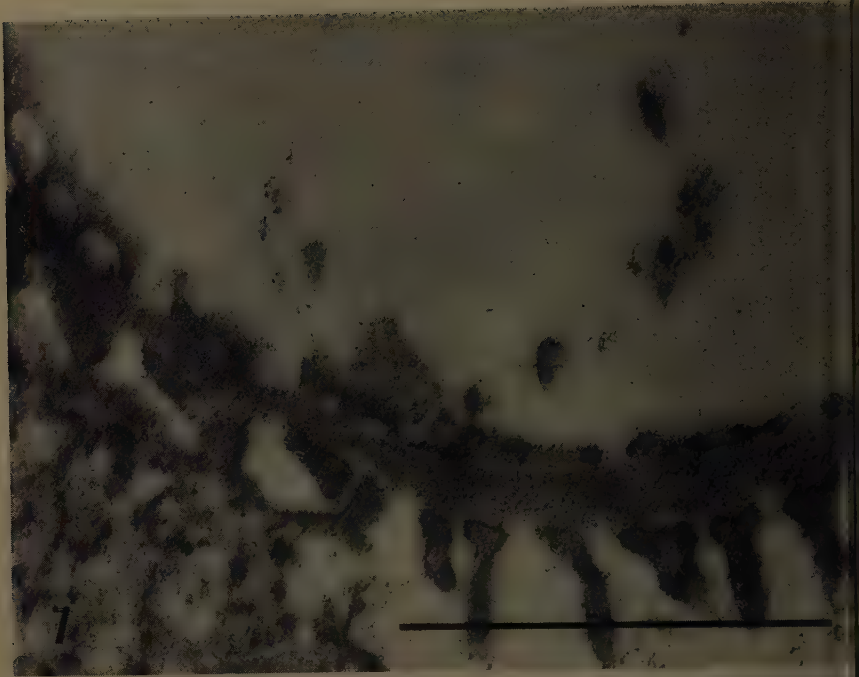


FIGURE 5. Electron micrograph of the striated border of a principal cell on a villus of the duodenum of a use. The processes of the border are tightly packed and of the same height and diameter. The surface mem-ne of the processes appears granular and thus different from ordinary cell membranes. Extending downward the cytoplasm from between the bases of the processes are coiled tubules similar to those of the proximal ule cells of the kidney (arrow). Chrome-osmic fixation. (pH 7.2). Approximately ×57,000.

FIGURE 6. Electron micrograph of portions of two S-37 tumor cells grown in peritoneal fluid. The lower cell division, the upper one is not. Cell processes cut at various angles are seen at the border of the lower cell. y vary in length, diameter, spacing, and orientation. Chrome-osmic fixation (pH 7.2). Approximately ,000.



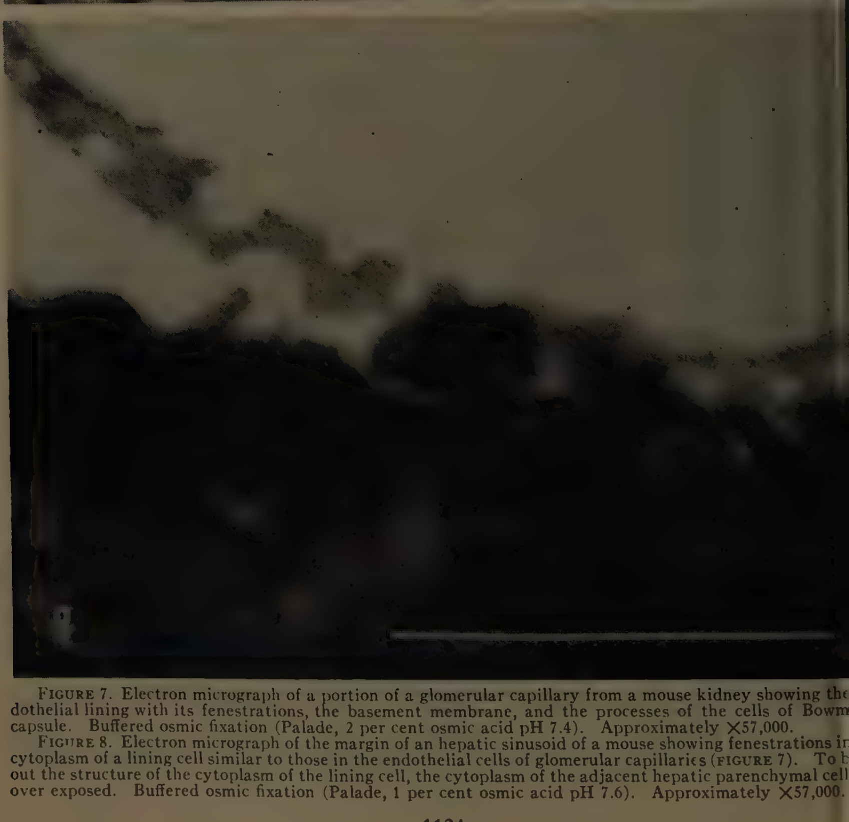
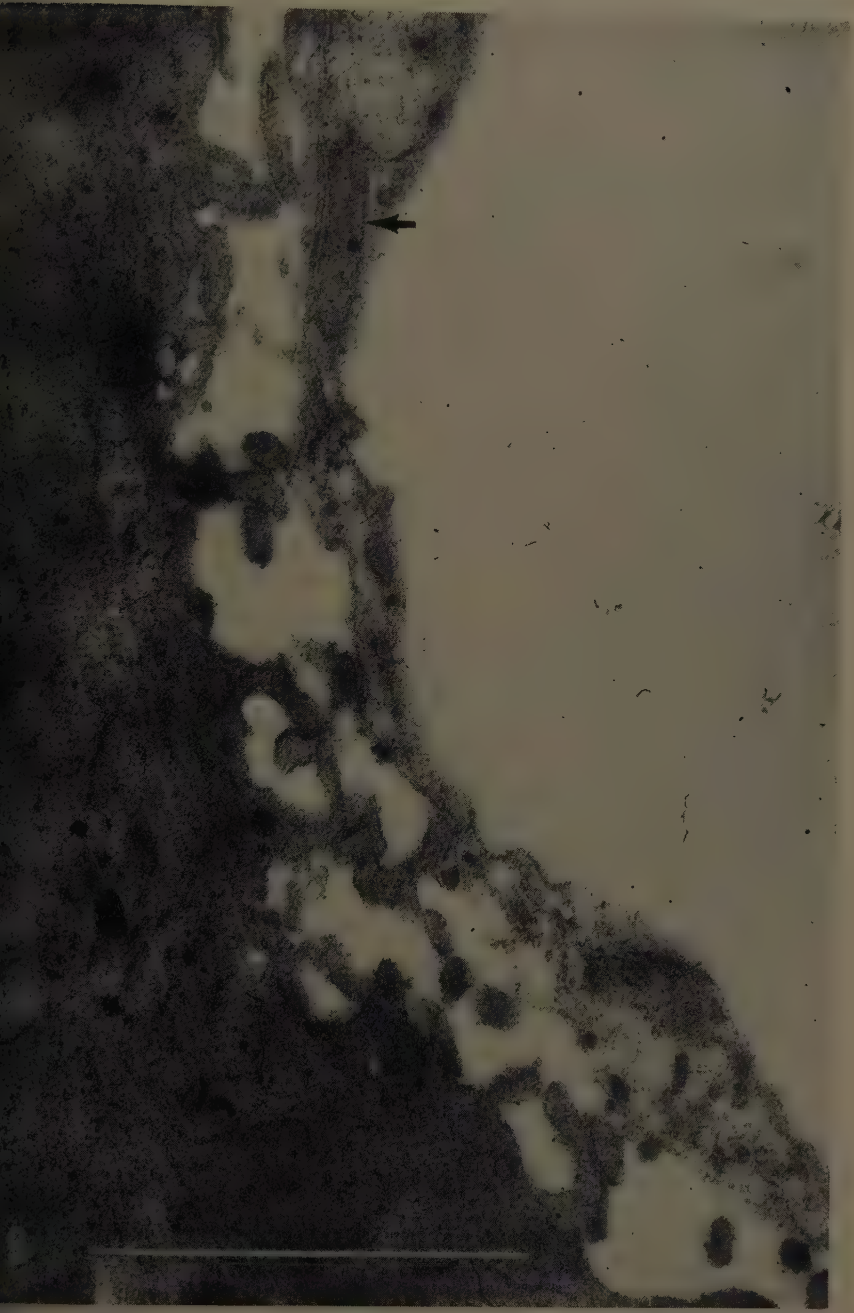


FIGURE 7. Electron micrograph of a portion of a glomerular capillary from a mouse kidney showing the dothelial lining with its fenestrations, the basement membrane, and the processes of the cells of Bowm capsule. Buffered osmic fixation (Palade, 2 per cent osmic acid pH 7.4). Approximately ×57,000.

FIGURE 8. Electron micrograph of the margin of an hepatic sinusoid of a mouse showing fenestrations i cytoplasm of a lining cell similar to those in the endothelial cells of glomerular capillaries (FIGURE 7). To b out the structure of the cytoplasm of the lining cell, the cytoplasm of the adjacent hepatic parenchymal cell over exposed. Buffered osmic fixation (Palade, 1 per cent osmic acid pH 7.6). Approximately ×57,000.

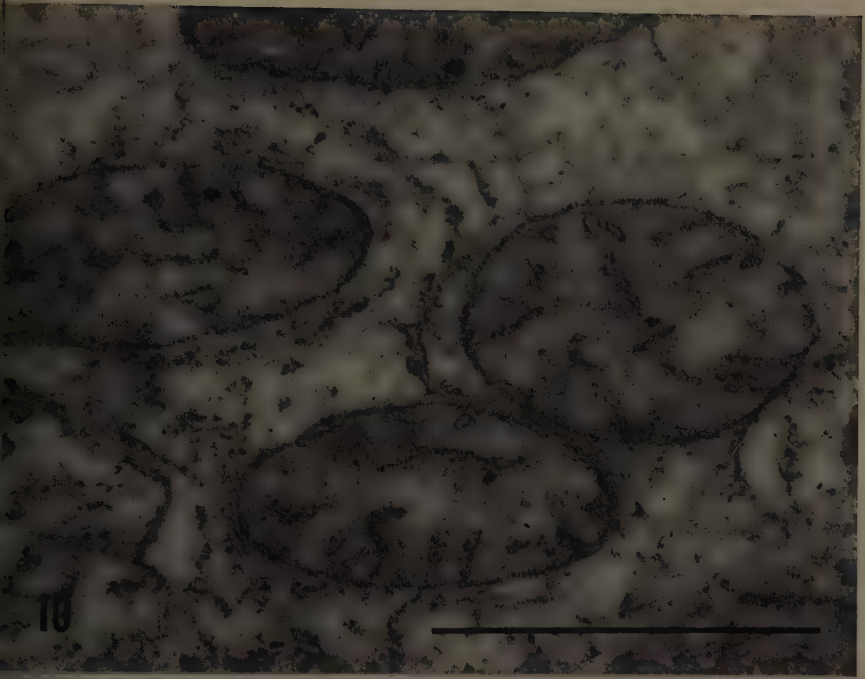


IGURE 9. Electron micrograph of the margin of an hepatic sinusoid from a mouse different from that from n FIGURE 8 was taken showing the cytoplasm of a lining cell. No fenestrations are evident. Mitochondria e lining cells (arrow) are frequently only 0.1 μ in diameter, considerably below the resolving power of the microscope. Chrome-osmic fixation (pH 7.2). Approximately ×57,000.

when a spectrum of animal cell types is examined. Mitochondria of hepa
cells possess relatively few and incomplete internal membranes[18] (FIGURE 1
while those of cells of the proximal convoluted tubules of the kidney are n
merous, closely packed, and extend completely across the width of the mi
chondrion[19] (FIGURE 11). FIGURE 11 shows a considerable variation in t
thickness of the membranes in a single mitochondrion. Osmiophilic granu
are characteristic of the mitochondria of many cell types, *e.g.*, those of t
proximal tubule cells of the kidney, hepatic parenchymal cells, and princip
cells of the duodenum, but have not been found in mitochondria of other c
types, *e.g.*, plasma cells. Before turning to a consideration of other cell co
ponents, it should be mentioned that mitochondria vary considerably in size
from the 0.3 μ diameter of those of proximal tubule cells of the kidney (FIGU
11) to the less than 0.1 μ diameter of some of the mitochondria of von Kupf
cells of the liver (FIGURE 9), these latter being too small to be clearly resolv
with the light or phase-contrast microscopes.

No other cell component has become so innocently involved in seman
controversy as the ergastoplasm or basophilic substance of cells. Porter a
Thompson in 1947[20] first described its electron microscopy in whole mou
of tissue culture cells, and since it appeared as a branching reticulum in t
endoplasm of these cells, they called it the "endoplasmic reticulum." In 19
we first described it in sections of hepatic and hepatoma cells and referred
it as the basophilic substance.[21] We suggested at that time that the filamen
later identified as lamellae,[22] were possibly comparable to the structures
scribed by Porter and Thompson. Recently, Palade and Porter[23] have verif
this. Shortly after our first description, Bernhard *et al.*[24] independently
scribed it in hepatic and salivary gland cells and homologized it with the ergas
plasm of Garnier.[25] More recently, Sjöstrand[19] described it as "the dou
membrane system" of cells. Palade[26] and, shortly thereafter, Sjöstrand a
Rhodin[27] demonstrated that small (15 mμ) granules of high electron scatter
power are usually associated with the membranes of the ergastoplasm. Pala
and Porter[23] showed that the membranes are sometimes free of granules a
conversely, Bernhard *et al.*[28] called attention to the fact that, in some speciali
cell types, the granules may be free in the cytoplasm in the complete abse
of the membranes. Palay and Palade[29] recently showed that a compara
situation may exist in the Nissl substance of some nerve cells. Weiss[30] cal
attention to the extreme sensitivity of the ergastoplasmic membranes to va
tions in tonicity of the cell environment and referred to the double membra
and their associated granules as "ergastoplasmic sacs." It is clear that
of these investigators have been studying the same cell component or com
nents, using different terminology in referring to it. At this point, we sho
like to insert one example of this component for later comparison with
counterpart in neoplastic cells. FIGURE 12 shows the ergastoplasm of a p
creatic cell with its double membranes and small granules.

While there is considerable evidence to support the view that the sm
granules of Palade are primarily responsible for the basophilic staining of cy
plasm, the double membranes themselves, which are actually profiles of f



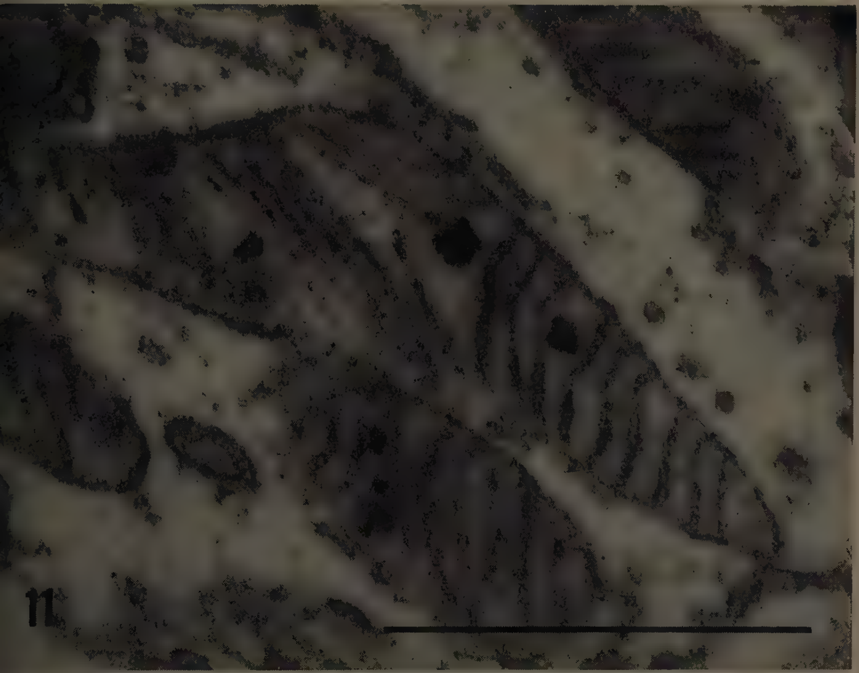


FIGURE 10. Electron micrograph of mitochondria of an hepatic cell of a rat showing the arrangement and
ative number of the internal membranes. The animal from which this specimen was taken was fasted five
ys, then given food 16 hours before being sacrificed. Note the relative scarcity of the ergastoplasmic mem-
anes in the cytoplasm. Chrome-osmic fixation (pH 7.2). Approximately ×57,000.

FIGURE 11. Electron micrograph of a mitochondrion from a proximal convoluted tubule cell of a mouse kid-
. The internal membranes extend from one border of the mitochondrion to another. Those membranes in
distended part of the mitochondrion appear to be stretched and are thinner than those of the narrower por-
n to the right. Chrome-osmic fixation (pH 7.2). Approximately ×57,000.

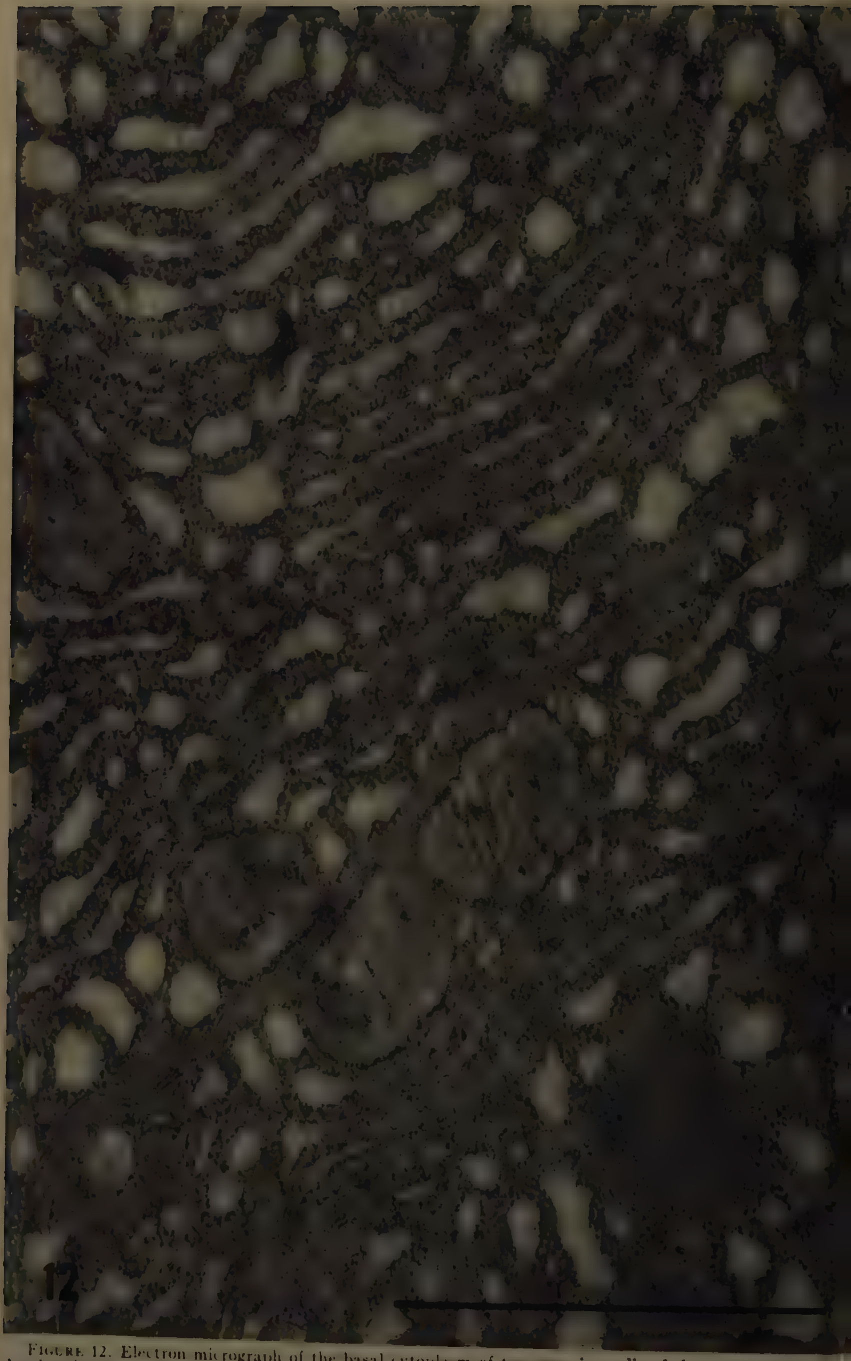


FIGURE 12. Electron micrograph of the basal cytoplasm of two exocrine cells of the pancreas of a mo
showing the ergastoplasm with its membranes and small (Palade) granules. Zymogen granules are visible at t
lower right. The cell boundaries of the two cells extend from the lower left diagonally to the up
right. Chrome-osmic fixation (pH 7.2). Approximately ×57,000.

ned sacs, are involved in the development of the striated or lamellated ap-
earance of the ergastoplasm as originally described by Garnier[25] with the light
icroscope. On the other hand, the name "endoplasmic reticulum," a per-
ctly good descriptive term for the ergastoplasm in the original studies by
orter, does not accurately describe its form and location in many functional,
ighly specialized cells where endoplasm cannot be distinguished from ecto-
lasm and where it has not been clearly established that a reticulum of double
embranes is consistently present.

The reality of the Golgi substance and the faithful preservation of its form
y classical methods of visualization have been supported by the majority
nd questioned by a minority of cytologists over a period of many years. The
asons for this continuing controversy were, in part, the difficulty of visualiza-
on in living, unstained cells, and also the fact that the size of the component
arts of the complex were below the resolving power of the light microscope.
ecently, we demonstrated that the Golgi substance, in essentially its classical
rm, could be seen with the phase-contrast microscope in freshly isolated,
nstained cells of the epididymis. Electron micrographs of the Golgi zone
these cells revealed a complex consisting of large vacuoles surrounded more
less by a system of smooth double membranes and small (40 mμ) granules
vesicles. Evidence was presented suggesting that the granules were derived
om a budding-off from the double membranes.[31] More recently, one of us
owed that this complex blackens preferentially with osmic acid after appropri-
e treatment.[32] FIGURE 13 is of the Golgi zone of a principal cell of the duo-
enum. It shows the three components of the Golgi complex arranged in a
rm comparable to that in cells of the epididymis. FIGURE 14, also of the
olgi zone of a principal cell of the duodenum, shows the complex after sec-
ndary treatment with osmic acid. Since this demonstration, several com-
unications have appeared containing evidence that this ultrastructure of the
mplex is characteristic of a variety of normal cell types. Thus the electron
icroscope, in conjunction with the phase-contrast microscope, has contributed
rong evidence supporting the classical view of the reality of the Golgi sub-
ance and has revealed details of its ultrastructure.

With the exception of secretory granules and cell inclusions such as lipid
oplets and pigment granules that are of variable occurrence, we have de-
ribed the fine structure of the nuclear and cytoplasmic components at present
entifiable in interkinetic normal cells. These descriptions should serve as a
sis for comparison with similar components of neoplastic cells.

Turning now to this comparison, no significant differences in the structure
nuclei of neoplastic cells have come to light thus far. Nuclei possess the
uble membrane characteristic of normal cells (FIGURE 15). Nucleoli may
larger and more numerous but their detailed structure is the same. The
all 15 mμ granules are also characteristic of nucleoli of neoplastic cells
IGURE 16). Alignment of granules in association with very thin membranes
so is characteristic of the chromatin of the interkinetic nuclei of neoplastic
lls (FIGURE 17). In dividing cells, we have found no evidence of a membrane
parating chromosomes from the cytoplasm. There is, however, a layer of
all 15 mμ granules forming the boundary between chromosomes and cyto-

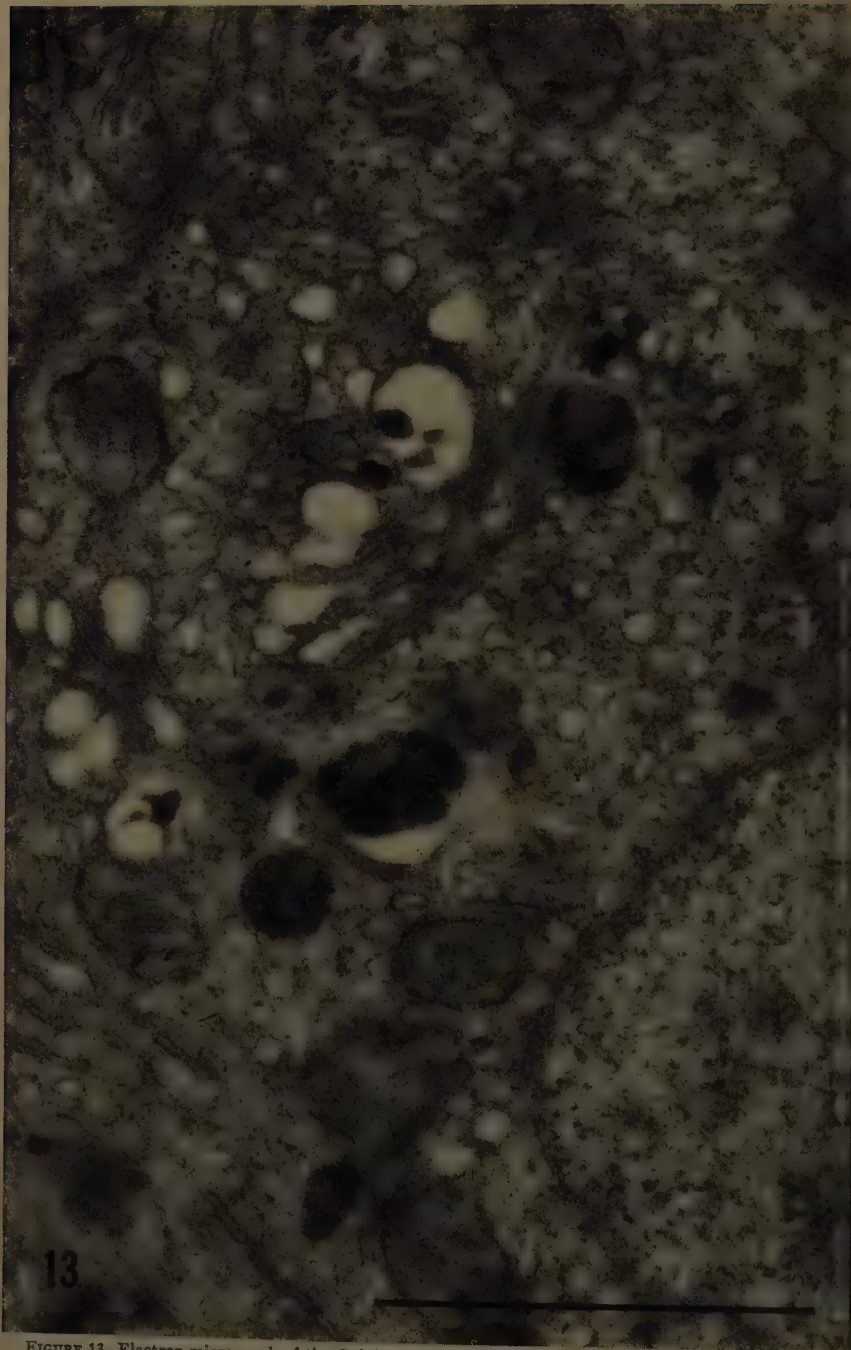


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FIGURE 13. Electron micrograph of the Golgi zone of a principal cell of the duodenum of a mouse show the Golgi complex. It consists of large vacuoles (some containing variable numbers of small osmiophilic gr ules), smooth double membranes marginating the vacuoles, and clusters of small granules or vesicles possess a periphery of high electron scattering power and an electron lucent center. Three lipid droplets are pres between the Golgi complex and the nuclear border. Chrome-osmic fixation (pH 7.2). Approximately ×57,

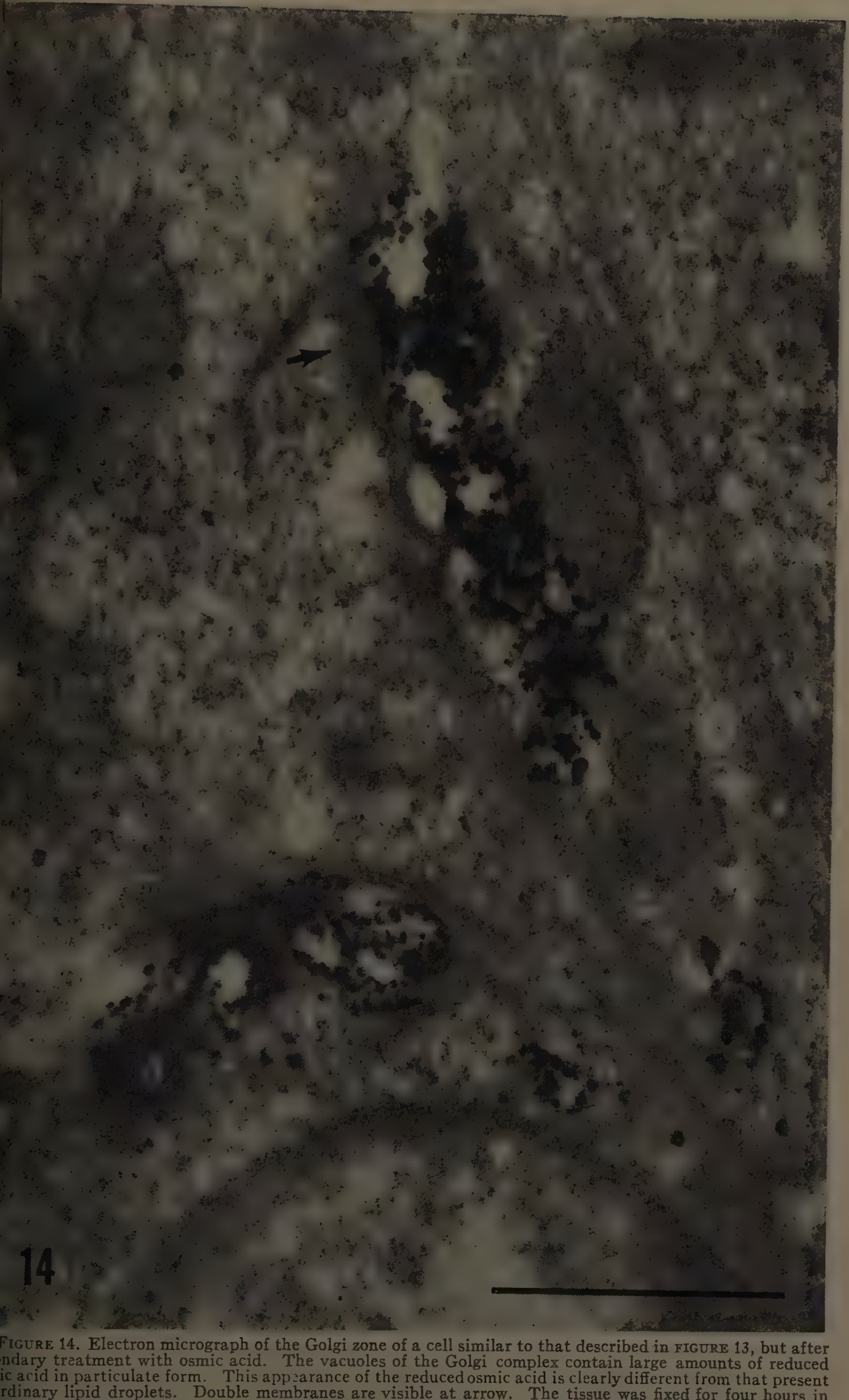


Figure 14. Electron micrograph of the Golgi zone of a cell similar to that described in FIGURE 13, but after
ndary treatment with osmic acid. The vacuoles of the Golgi complex contain large amounts of reduced
ic acid in particulate form. This appearance of the reduced osmic acid is clearly different from that present
rdinary lipid droplets. Double membranes are visible at arrow. The tissue was fixed for four hours in
de's veronal-acetate buffered osmic acid (pH 7.4), washed one hour in distilled water, and immersed in 2 per
osmic acid at 37° C. for 18 hours. This treatment results in considerable swelling of the tissue, so that the
ponent parts of the complex appear to be larger than those in FIGURE 12. Approximately ×39,000.

FIGURE 15. Electron micrograph of a nucleus of an S-37 tumor cell grown subcutaneously showing the do
nuclear membrane. Chrome-osmic fixation (pH 7.2). Approximately ×72,000.

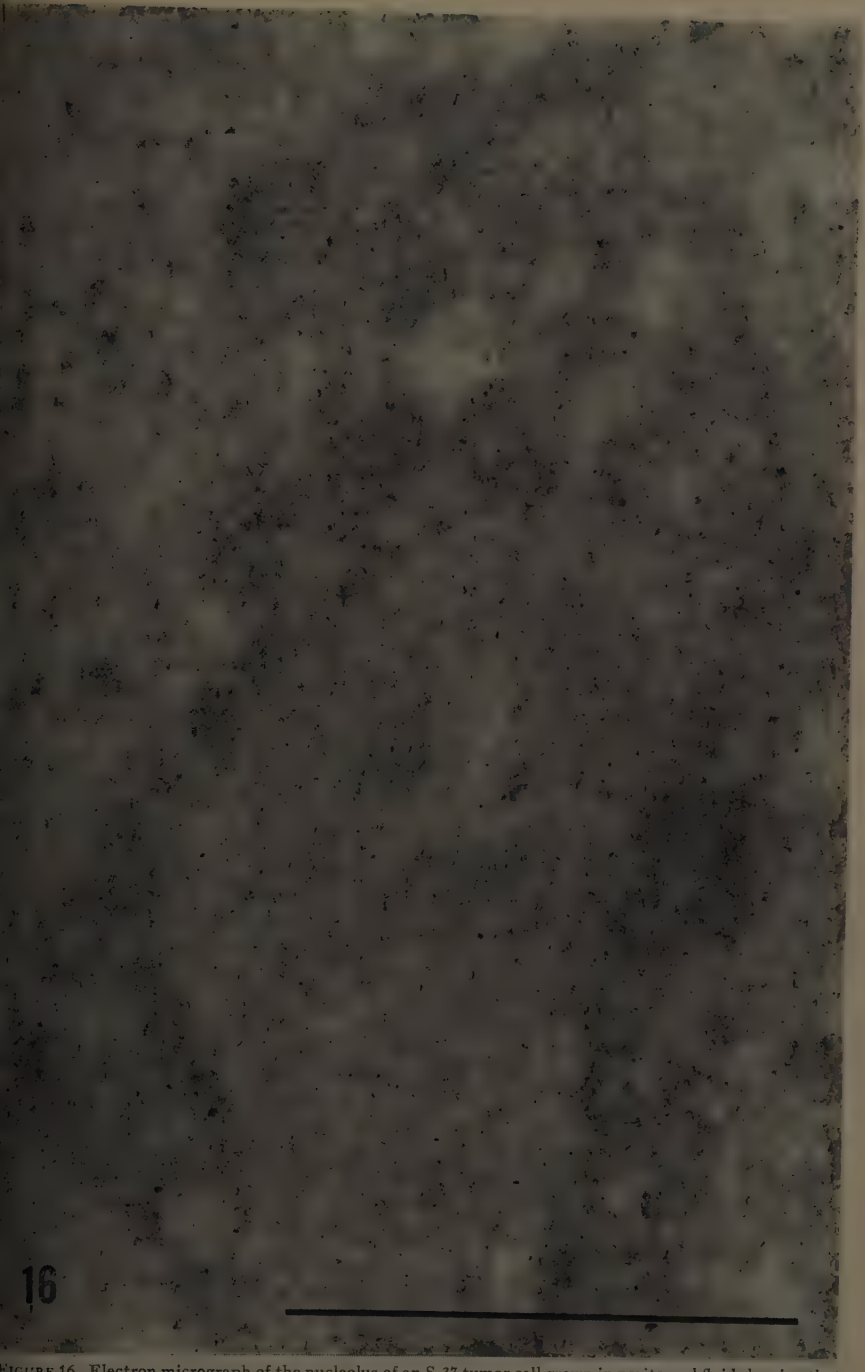


FIGURE 16. Electron micrograph of the nucleolus of an S-37 tumor cell grown in peritoneal fluid showing that apparent density of its outer border is the result of the presence of numerous small granules comparable to granules of the ergastoplasm. In the lower center of the figure is a large lighter area containing closely anastomosing strands with a diameter of approximately $7\ m\mu$. Chrome-osmic fixation (pH 7.2). Approxi-ely ×67,000.

17

Figure 17. Electron micrograph of the nucleoplasm of the nucleus of an S-37 tumor cell grown subcut
ously. Alignment of small granules along thin membranes is present (arrows). The small dark granule
thought to be deposits of chromium. Chrome-osmic fixation (pH 7.2). Approximately ×67,000.

lasm. Whether these granules are derived from the cytoplasm or nucleus
as not as yet been determined. It is suspected that a similar distribution of
ranules occurs during mitosis in normal cells.

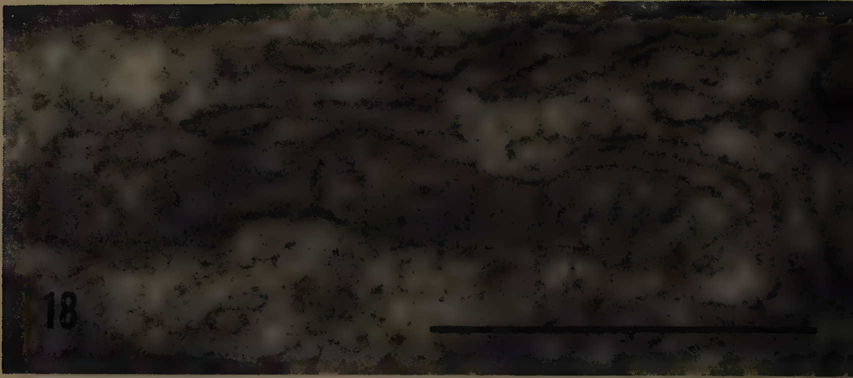
Minor differences between the cytoplasmic components of normal and malig-
ant cells have been noted. Mitochondria possess the same basic structure
both but, in neoplastic cells, the internal membranes tend to be less regularly
riented than in normal cells (FIGURE 18). There are exceptions to this, how-
ver, since Howatson and Hamm[33] have shown that the internal membranes
the mitochondria of hepatoma cells show more regular orientation than do
ose of normal hepatic cells. Bizarre-shaped mitochondria are more fre-
uently found in neoplastic cells than in normal cells but, again, there are
any exceptions to this. The osmiophilic granules characteristic of the mito-
ondria of many normal cells are usually inconspicuous in, and sometimes
bsent from, those of neoplastic cells.

The ergastoplasm of neoplastic cells is usually less in amount than in normal
ells, with fewer ergastoplasmic membranes. In addition, the small granules
Palade are not consistently associated with the membranes, many of them
ppearing free in the cytoplasm (FIGURE 19). This, again, is a quantitative
ifference and is principally true of cells of rapidly growing tumors.

Tumor cells possess all three components of the Golgi complex, but the vacu-
lar component is usually less conspicuous than in the majority of normal
ells (FIGURE 20). Again, there are exceptions, as in the case of melanoma S-91,
which the Golgi complex, including the vacuolar component, is highly de-
eloped and occupies a relatively large part of the cytoplasmic area (FIGURE
1). In this instance, of course, the melanoma cells are functioning in the
roduction of melanin granules. Haguenau and Bernhard[34] have recently de-
ribed other exceptions.

Thus, in regard to the detailed structure of normally occurring components
the nucleus and cytoplasm, we have found no unequivocal evidence of quali-
tive differences between normal and malignant cells. Such a finding should
ardly be considered surprising, however. All of the components considered
ove are assumed to play a vital role in cellular metabolism and, since both
oplastic cells and the majority of normal cells are capable of metabolic ac-
vity, growth, and division, it would be surprising indeed if these various com-
onents differed markedly in the two cell groups.

We have found only one qualitative difference between the series of normal
d the series of neoplastic cells we have examined. This difference has been
e presence of extracellular or intracytoplasmic particles of the size range of
ruses in some, but not all, tumors examined. FIGURE 22 shows the extra-
llular position of particles associated with subcutaneous transplants of S-37.
hese particles were not found associated with S-37 grown in peritoneal fluid.
IGURE 23 shows particles of similar size present in a specialized area of cyto-
asm of a cell of melanoma S-91. We have not found comparable particles
sociated with a transplantable lymphoma nor with a transplantable hepa-
ma. If these particles, when present, actually represent virus, we should
efer to consider them as vicariously acquired some time during the many
nerations of tumor transplants.



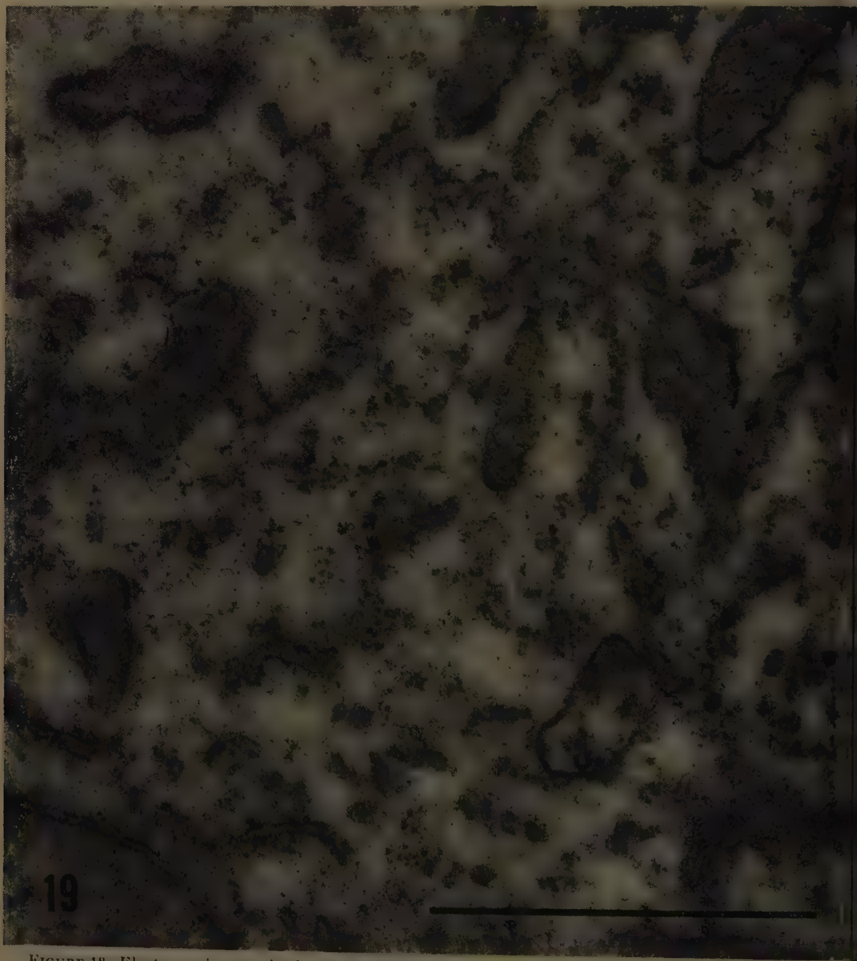
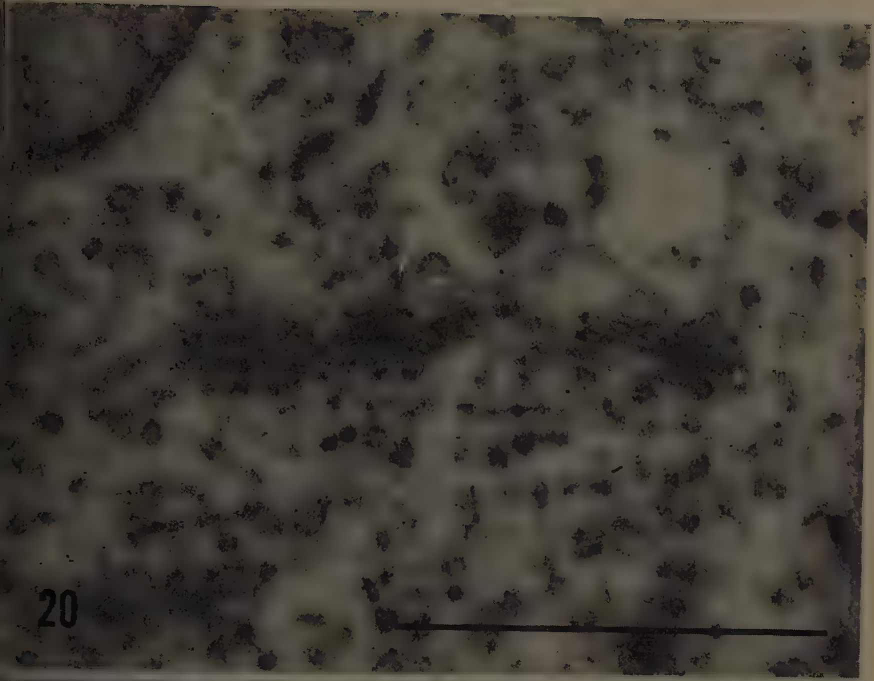


FIGURE 18. Electron micrograph of a mitochondrion of a Rous sarcoma cell showing irregularity of orier
tion of the internal membranes. Chrome-osmic fixation (pH 7.2). Approximately ×50,000.

FIGURE 19. Electron micrograph of the cytoplasm of an S-37 tumor cell grown subcutaneously showing
small granules of Palade, some associated with the membranes of the ergastoplasm, some appearing free in
cytoplasm. A small part of the nucleus is at the lower left. Chrome-osmic fixation (pH 7.2). Approxima
×50,000.



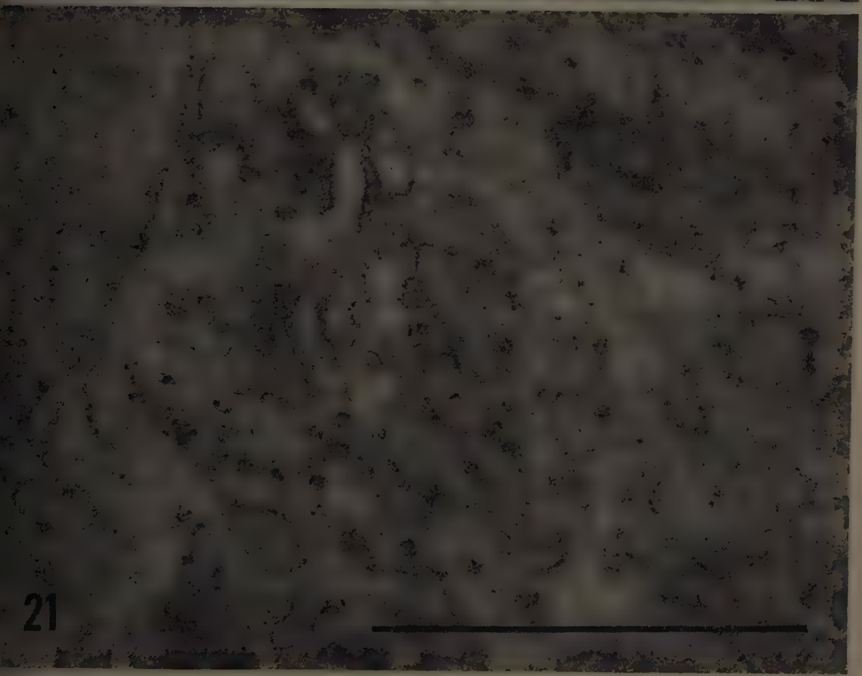
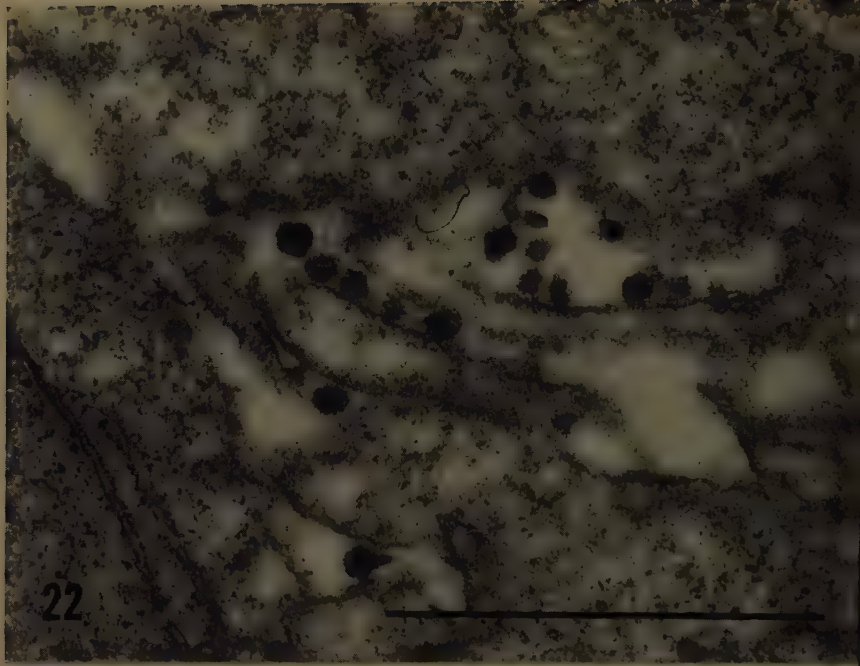


FIGURE 20. Electron micrograph of the Golgi area of an S-37 tumor cell grown in peritoneal fluid showing ups of smooth double membranes and clusters of small granules making up the Golgi complex. A small part the nucleus is at the upper left. Chrome-osmic fixation (pH 7.2). Approximately ×57,000.

FIGURE 21. Electron micrograph of part of the cytoplasm of an S-91 melanoma cell grown subcutaneously wing an extensive Golgi complex with vacuoles, groups of double membranes and clusters of small granules. t of the nucleus appears at the upper left. Chrome-osmic fixation (pH 7.2). Approximately ×57,000.



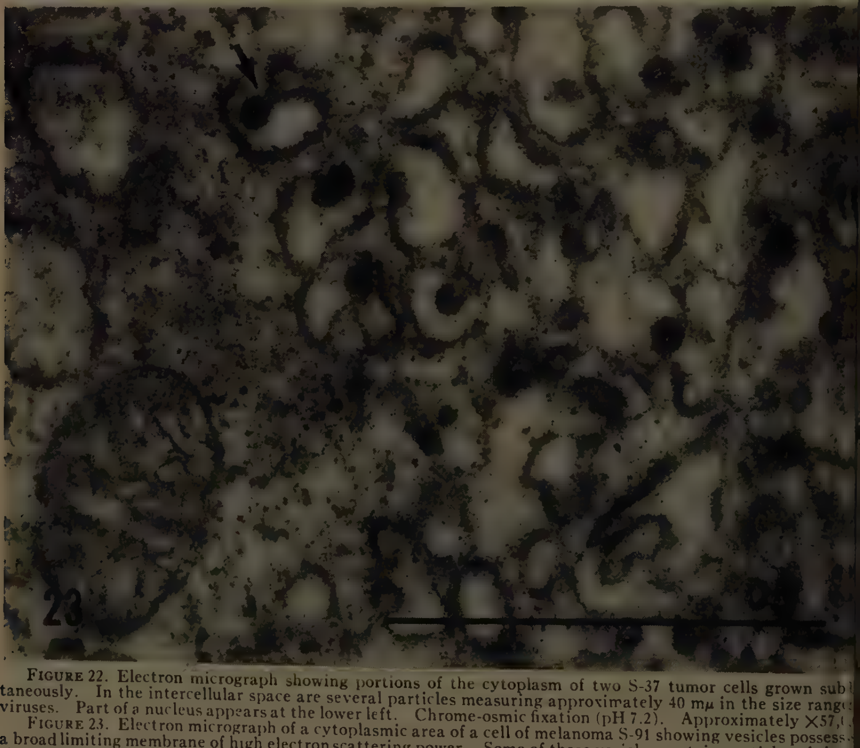


FIGURE 22. Electron micrograph showing portions of the cytoplasm of two S-37 tumor cells grown sub taneously. In the intercellular space are several particles measuring approximately 40 mμ in the size range viruses. Part of a nucleus appears at the lower left. Chrome-osmic fixation (pH 7.2). Approximately ×57,

FIGURE 23. Electron micrograph of a cytoplasmic area of a cell of melanoma S-91 showing vesicles possess a broad limiting membrane of high electron scattering power. Some of these vesicles contain particles of sim size to those in FIGURE 22. Occasionally these particles show attachment to the limiting membranes of vesicles (arrow). No evidence has been found to indicate that these particles are associated with the format of melanin granules. A spherical mitochondrion is visible at the lower left. Chrome-osmic fixation (pH 7 Approximately ×57,000.

We might summarize this discussion by suggesting that the quantitative differences noted between normal and neoplastic cells are probably related to differences in such factors as metabolic activity and growth rate, while the qualitative difference is related to an extrinsic factor. Neither of these differences appear to be basically related to the existence of the neoplastic state itself.

Obviously, the application of electron microscopy to problems of neoplasia is in its infancy, but we should like to suggest the probability that there are more fertile fields for its use than the one just described. Such an area might be the study of possible modifications in fine structure of neoplastic cells during long-term exposure to controlled but varied environmental conditions.

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Part III. Specialized Developments in Cytochemistry

APPLICATIONS OF SOFT X-RAY METHODS TO A STUDY OF NORMAL AND NEOPLASTIC CELLS*

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1. *Introduction*

In 1950, Engstrom and Lindstrom described an ingenious method whereby they could measure the amount of mass (substance) present in very small biological specimens such as a histologic section, or even a cell. They utilized the fact that the absorption of soft X rays is determined by the mass present. The more mass present, the greater the absorption of X rays. The absorption of X rays was determined by placing the specimen on photographic emulsion and exposing the combination to a particular X ray beam, thus taking a roentgenograph or microradiograph of the object. Considerable histologic and cytologic detail was obtained. Furthermore, by measuring the different amount of darkening (density) produced in the emulsion, one could determine the transmission of X rays and thereby estimate the amount of absorbing mass present in the object. This was achieved by comparing the densities obtained in the emulsion beneath the specimen to that produced by standards containing known amounts of similar material and simultaneously exposed to the same X-ray beam (FIGURES 1, 2, 3). Masses of the order of 10^{-13} to 10^{-10} gm. were accessible to measurement.[1]

Since the technique has been described in detail by Engstrom[2, 3] and others,[4-10] only those features not previously reported will be emphasized.

2. *Theory*

(a) *Absorption of X rays.* The absorption of monochromatic X rays is represented by the equation

$$I = I_o e^{-\mu d} \qquad (1)$$

where I = transmitted intensity
I_o = incident intensity
e = base of the natural system of logarithms
d = thickness of absorbing substance (cms)
μ = linear absorption coefficient (logarithm of the fractional decrease in intensity, per cm.)

Since I would vary with the specific gravity of the absorbing substance, an expression that would correct for this factor and be independent of density is preferred, and the mass absorption coefficient $\left(\frac{\mu}{\rho}\right)$ is used. ρ is the density By substituting the simple relationship

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* This study was supported by a grant from the Damon Runyon Memorial Fund for Cancer Research, New York, N. Y. The author is much indebted to the firm of Williams and Sherman, Bloomfield, N. J., who designed and built the equipment and to Drs. A. Engstrom, J. Clemmons, and H. Beyer for many valuable suggestions. The project was initiated and developed at the Pathology Laboratories and the Department of Physics and Biophysics of the Sloan-Kettering Institute, Memorial Center for Cancer and Allied Disease, New York, N. Y.

$$d = \frac{m}{\rho} \qquad (2$$

in EQUATION 1 where d = thickness
ρ = density
m = mass per unit area

$$I = I_0 e^{-(\mu/\rho)\cdot(m)} \qquad (3$$

Subsequent use of the term mass is understood to mean mass per unit area These equations also hold for X-ray beams that are not monochromatic whe they are not too widely divergent, as in the spectrum used in this techniqu

The mass absorption coefficient $\frac{\mu}{\rho}$ is composed of two parts: *a mass phot electric absorption coefficient*, $\frac{\tau}{\rho}$, representing the energy diverted from the incide beam to the production of characteristic X rays and photoelectrons; and th *mass-scattering coefficient*, $\frac{\sigma}{\rho}$, which represents the of energy-producing un modified scattered X rays, modified scattered X rays, and recoil electrons, *i.*

$$\frac{\mu}{\rho} = \frac{\tau}{\rho} + \frac{\sigma}{\rho} \qquad (4$$

The mass photoelectric absorption coefficient

$$\frac{\tau}{\rho} = CZ^4\lambda^3 \qquad (5$$

where C = constant between critical absorption wave lengths
Z = atomic number of absorber
λ = wave length of incident X rays

It can be seen, therefore, that photoelectric absorption is large for heav elements and long wave lengths, and small for light elements and short wav lengths. In the method to be described, very long wave-length X rays ar low numbered atom elements are involved. Since the mass scattering is numerically very small in comparison to the photoelectric absorption at the wav length X rays employed, its effect is ignored, and the absorption is considere entirely photoelectric.[2]

It is more convenient to work with natural logarithms, so that EQUATION is expressed as

$$ln\,\frac{I_o}{I} = \frac{\mu}{\rho}\cdot m \qquad (6$$

(b) *X-ray image formation.* In the method under discussion, a one-to-o shadow image of the object is taken on fine-grain emulsion, the resulting micr

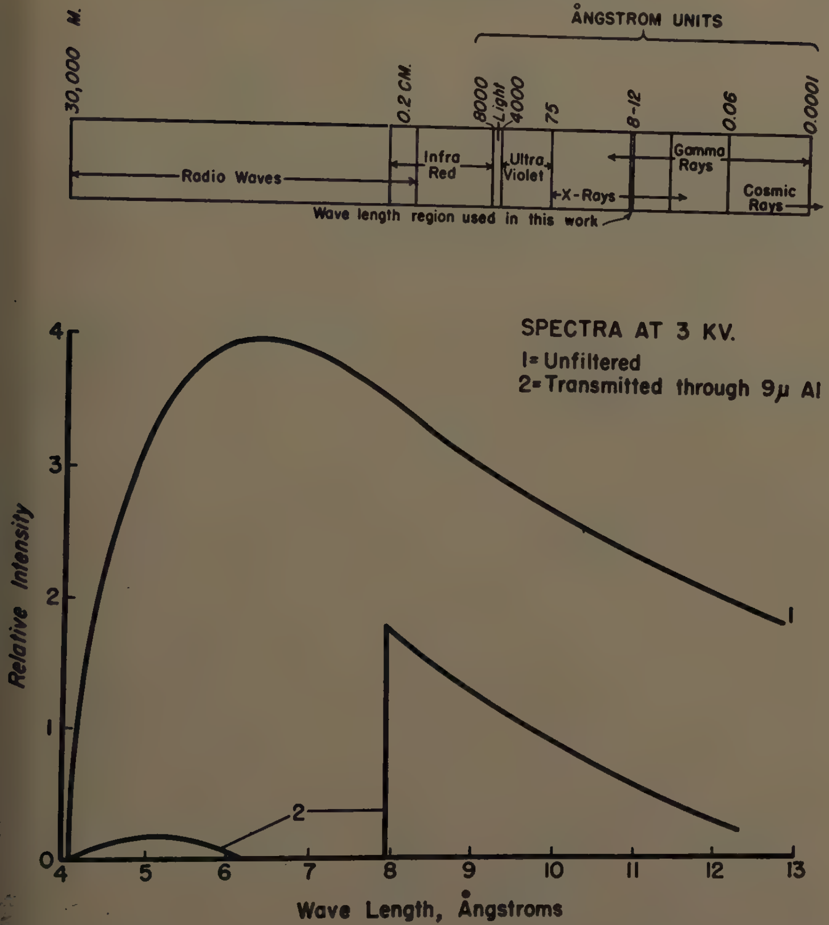

FIGURE 1. Calculated X-ray spectrum used in mass determination method and relation to electromagnetic cale.

adiograph is developed, and then may be viewed or photographed through he visible light microscope.

The X rays are generated from a small focal spot and pass in a divergent eam to the specimen and to photographic film. Because the X rays are not ighly collimated, it is apparent that there will be some blurring or unsharpess to the image formed because of geometrical considerations. The imporant factors in the sharpness or blurring of the image will be the size of the ocal spot, the distance to the film, the thickness of the specimen, the proximity f the specimen to the film, and the resolving power and thickness of the photoraphic emulsion.[2]

Because of the use of line focusing, of a target inclination of 20° from the erpendicular to the electron beam, and of a filament length of 0.5 in., the unlterable length of our projected focal spot is 4.6 mm. Movement of the filament in and out of the focal cup will change the width of the spot and, althoug we have observed widths of the order of 0.5 mm., exposure times were prohib tive. Present adjustments produce a width of approximately 1 mm. Thi with a focal distance of 90 mm., gives an unsharpness of less than 0.25 micro We have obtained a clear image of a 0.5-micron wire but, usually, film gran larity is so pronounced at this level of magnification that such a demonstrati represents a limit of the technique not usually attained with biological object

(c) *Emulsion.* Gaevert Lippmann and Eastman Kodak 649-0 spectr scopic emulsions, each having resolving powers of about 1000 lines per mil meter, have been used. They permit magnifications up to about 500 to 60 times, occasionally higher. Recently, Eastman Kodak has prepared a 1 micron-thick 649-0 emulsion on a cellulose-acetate 35-mm. base that has prove very satisfactory.* Unless special precautions are taken, as with the 10-micro emulsion, dirt and various extraneous objects accumulate in the emulsi during its manufacture and in the performance of the technique, and they a an annoying and sometimes prohibitive factor.

(d) *The relationship of the mass of biological object, its composition, an X-ray absorption.* The nonaqueous portions of biological tissues consis from the quantitative aspect, mostly of protein or compounds with an eleme composition of carbon (C), nitrogen (N), oxygen (O), and hydrogen (H similar to that of protein. This is particularly true if the tissue has been fix and processed by the usual histologic techniques that dissolve much fat an carbohydrate. The percentage composition of these elements in protein relatively constant, averaging about 51 per cent C, 16 per cent N, 24 per ce O, and 7 per cent H. Within certain limits, the mass absorption coefficien of the C, N, O, and H have a constant ratio to each other at various wa lengths, so that the use of a narrow polychromatic spectrum of X rays is po sible. Calculations have shown that a 5-micron-thick biological object com posed mostly of C, N, and O, as in protein, must have a mass-absorption c efficient of about 2000 to give adequate X-ray absorption and good contras This requirement limits the use of X-ray measurements to wave lengths of to 50 Å (2.5 to 0.25 kv.). The X rays used (3 kv.) give a spectrum in whi about 85 per cent of the intensity lies in the long wave-length region of 8 12 Å.

The mass absorption coefficients of C, N, O, and H are high at the wa lengths used and, although they are not quite as high as the coefficients of sor other elements occurring in tissues, the product of their mass coefficients a their high concentrations determines that practically all the absorption X rays will be caused by the C, N, O, and H present (see EQUATION 6). D termination of the amounts of these elements present by X-ray absorption m lead directly to the calculation of the mass in view of the constant percenta composition of tissue. Even wide variations from the average compositi of protein produce little change in the total absorption of X rays by biologi tissues, *e.g.*, C may vary as much as 18 per cent, N up to 60 per cent, and up to 8.5 per cent from the average composition without causing a systema

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* The author is indebted to Doctor John Spence and the Eastman Kodak Co. for their kindness in prepar this emulsion.

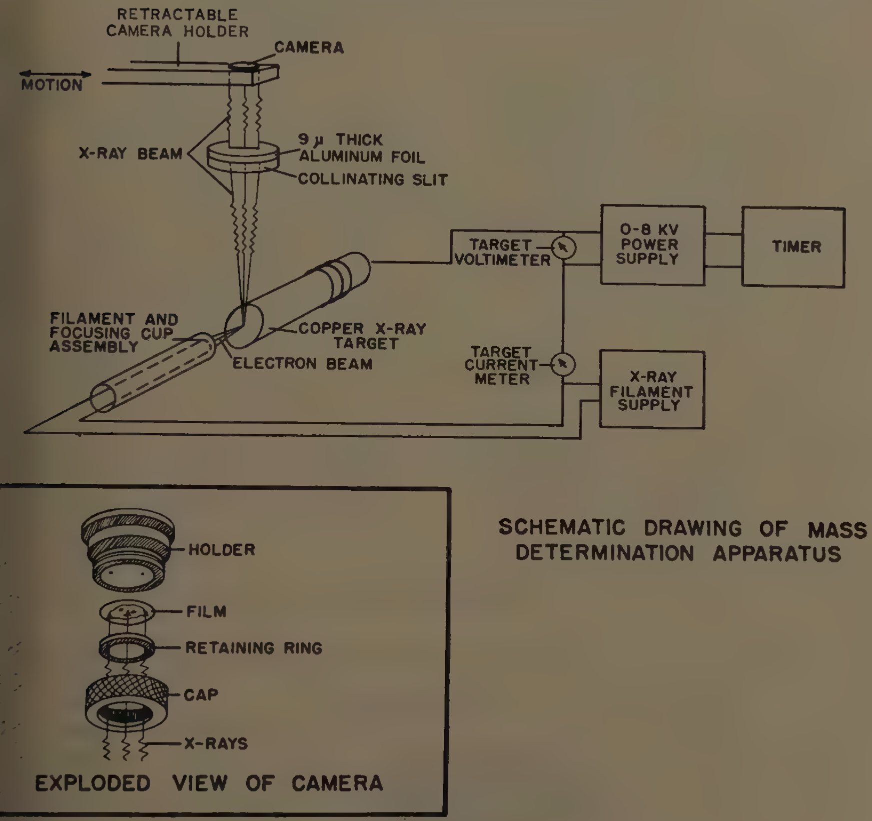


FIGURE 2. Schematic representation of basic units in the mass determination technic.

rror greater than 5 per cent (TABLE 1).[1] The presence of heavy elements ould introduce large error even in low concentrations, but they are not normally found in high enough concentration in biological tissues to affect significantly the absorption of X rays. Hydrogen absorbs disproportionately to ts concentration, and a correction factor for this is used. For objects with n unusually high concentration of an element, such as bone with its calcium, dditional correction factors and alterations of techniques are required.

TABLE 1

PERMISSIBLE VARIATION FROM PROTEIN OF AVERAGE COMPOSITION (C, 51%; N, 16%; O, 24%; H, 7%; AND S, 2%) TO KEEP ERROR IN MASS DETERMINATION 5% OR LESS

Iydrogen	5.3	Phosphorus	5.1
arbon	17.7	Sulfur	14.2
itrogen	59.8	Chlorine	17.3
xygen	8.7	Potassium	15.3
odium	2.4	Calcium	9.5
agnesium	2.6	Iron	2.9

(e) *Reference standards.* Since only the initial absorption of X rays is given by EQUATION 3, reference standards have to be used (FIGURES 3, 4). It is known that when the transmission of X rays through two samples of identica composition is equal, their masses are equal. Thus, by using standards o the same composition as the specimen and exposing them to the same X rays as those striking the specimen, one may plot the density produced in the photographic emulsion by the standards against the mass and obtain a curve from which one may interpret similar densities produced by the specimen (FIGURE 3). The standards can be used as an indicator of the gamma of the emulsion and also to show photographic processing abnormalities, fluctuation of X-ray intensity, and other undesirable conditions.

Calibration of Reference Standards

The reference system used by Engstrom and Lindstrom was composed of nitrated cellulose of the following composition: C, 47 per cent; N, 7 per cent; O, 41 per cent; and H, 5 per cent. Hydrogen gives a systematic error of about −7.5 per cent. The general expression for the mass (m) is

$$m = {}_{m}\mathrm{CNO} + mx \qquad (7)$$

where ${}_{m}\mathrm{CNO}$ = mass of CNO protein

mx = mass of any element other than CNO

If a step in the reference system gave the same absorption as the object unde

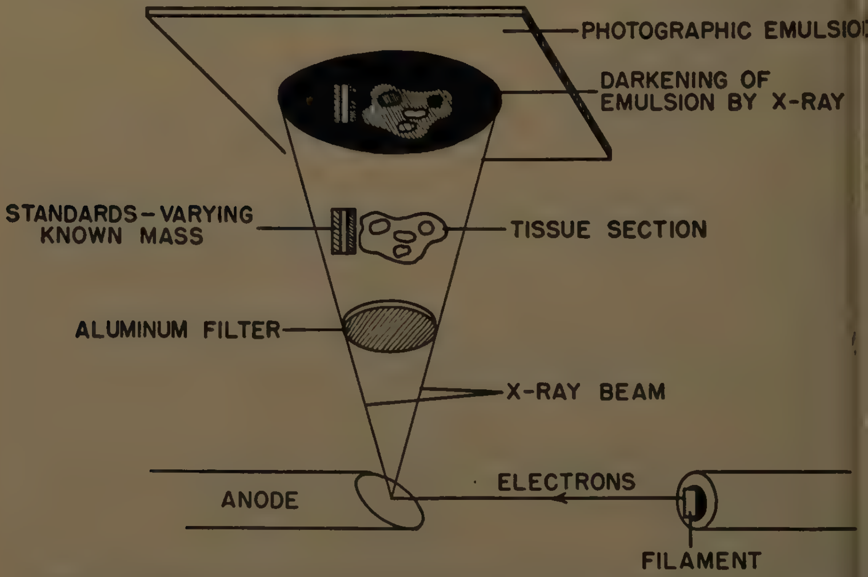


FIGURE 3. Schematic representation of the mass determination technique. Biological specimen on photographic emulsion absorbs X rays depending on its mass. Unabsorbed X rays darken emulsion. If tissu absorbs X rays completely, underlying emulsion is unaffected and is white. Amount of darkening (density) emulsion is indicative of amount of X rays passing through tissue and can be determined quantitatively by comparing with darkening associated with standards of known mass.

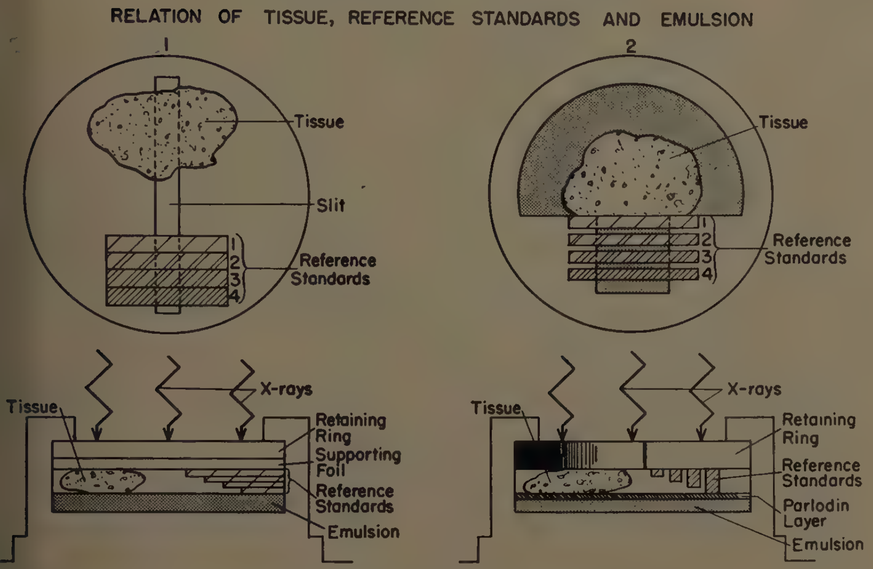


FIGURE 4. Relation of tissue, reference standards and emulsion: (1) the original method of Engstrom; (2) method we have used. Recently tissue has been placed directly on emulsion.[18]

measurement, then in terms of EQUATION 6 (and considering mx of EQUATION 7 as the mass of hydrogen) the comparison could be expressed as

$$ln \frac{I_o}{I} = k_H \cdot \left(\frac{\mu}{\rho}\right) \text{CNO} \cdot m = k_H \text{ ref} \cdot \left(\frac{\mu}{\rho}\right) \text{CNO ref} \cdot m \text{ ref} \quad (8)$$

where

k_H = correction factor for H of the CNO protein
CNO = protein of CNO
$k_H$ref = correction factor for H of reference
CNOref = CNO of reference
m ref = true mass of reference

Thus the true mass of the biologic structure (m) equals

$$m = \frac{k_H \text{ref}}{k_H} \cdot m \text{ ref} \cdot \frac{\left(\frac{\mu}{\rho}\right) \text{CNO ref}}{\left(\frac{\mu}{\rho}\right) \text{CNO}} \quad (9)$$

Lindstrom[11] determined the mass of the reference systems by gravimetric neans. Djurle and Hallén[12] and Brattgard[13] used interferometric methods to neasure the thickness of the standards and thereby decreased error.

Recently Clemmons[6] has found a dye, D and C green (alizarin green), that s uniformly absorbed by the parlodion reference system, and he calculates hickness of the reference step by measuring, with a densitometer, the absorption of light by the dye present in the standard. Multiple layers of dyed parlodion give absorption values consistent with the Lambert-Beer law.[14] W have used the latter method, and have also measured the dyed parlodion layer gravimetrically and by interferometry.

(f) *Estimation of errors.* Lindstrom considered the total error of th method to be ±26 per cent, and he pointed out that if weight per unit volum is calculated, this involves a further error in the measurement of thickness If two measurements of a tissue are made; *e.g.*, before and after an extractio procedure, then further error is added. If one is interested in relative differ ences, however, the error of the absolute weight of a reference step disappear and the error may be as low as ±12 per cent.[11] Brattgard *et al.* have measure the density of the areas in the microradiograph by projecting the enlarge image into a microphotometer, and they state that their error is only ±1. per cent. In determining the thickness of the reference standard interfero metrically, the error is said to be reduced to only ±1.5 per cent. If the Bratt gard-Hydén methods prove as successful as claimed, two of the largest source of error will have been reduced to negligible dimensions.[15]

Possible errors in measuring transmissions have been pointed out, as well a errors arising from inhomogenities in the specimen.[16, 17]

3. *X-Ray Equipment and Procedures*

(a) *X-ray tube.* The X-ray tube consists of four main parts, as shown i FIGURE 5: the block *A*; the anode arm *B*; the cathode arm *C*; and the super

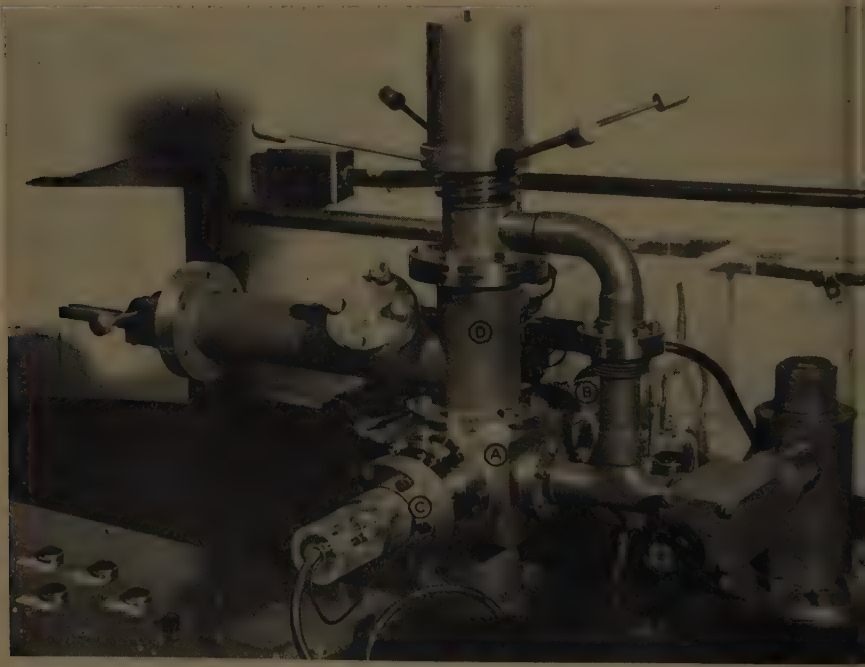


FIGURE 5. Photograph of X-ray tube. *A* is main block, *C* the cathode, *B* the anode, and *D* the superstructur

structure *D*. Leading off at a right angle is the camera-loading assembly with its port, through which the camera is positioned. The block *A* may be considered the envelope of the tube, to which all the other parts of the tube are bolted by means of gasketed flanges.

The anode arm *B* consists of two main parts: the anode assembly and the anode housing. The anode assembly has a highly polished copper target supported on a copper tube and brazed to a flange. A smaller diameter tube coaxial with the anode support, insures circulation of the water directly behind the target face.

The anode housing consists of three flanges. Two are joined together by means of a brass bellows, while the third is joined to one of them by a glass cylinder for electrical insulation purposes. A bellows arrangement makes possible the correct positioning of the target. The anode housing is bolted to the main block, and the anode assembly, in turn, is bolted to the housing. Both of these vacuum joints are made with neoprene "O"-ring gasket seals.

The cathode arm, *C*, houses the filament, its focusing cup, and a device for adjusting the filament in position. It is bolted on to the block in the same fashion as the anode arm. An adjustment allows rotation of the filament to align its emitting length with the axis of the focusing cup slit. The depth of the filament in this slit is controlled by a knurled collar adjustment, thus permitting control of the X-ray emitting spot size.

The filament is a 0.012-inch diameter tungsten wire bent into a rectangular U. The two legs thereby formed are each gripped by small kovar-pin vises and sealed into glass insulating supports. Filament connections to the hermetic seals are flexible copper leads insulated with glass beads. The hermetic seals and the external electrical connections are protected by a lucite cover.

The superstructure (FIGURE 6) consists functionally of two units: a horizontal member *A*, the camera arm, and the vertical member *B*, the main valve. Since very soft X rays are used, vacuum techniques are necessary, and the sample and the film must be placed inside the tube during exposure. During loading of the camera and exposure to the atmosphere, an air lock is used to permit continuous pumping of the X-ray tube proper.

The camera, with film and tissue sample, is introduced through a port (not shown) in camera arm *A*, with the valve plunger *C* seated on neoprene gasket *F*. The valve plunger is a hollow cylinder that permits communication between the upper (*I*) and lower portions (*J*) of the superstructure for vacuum lines but, when properly positioned, it seals off the camera arm. The plunger position is adjusted by rotating the valve collar *D* with the handles provided.

Exhaust lines *J* and *I* lead to a diffusion pump and insure that aluminum foil window *G* is equally pumped from both sides. After the camera arm *A* has been rough-pumped down to 50 μ Hg following specimen insertion, the valve plunger *C* is raised. The fore pump is excluded from the camera assembly arm, and the latter is further pumped to the necessary vacuum through *J* and *I* by the diffusion pump.

Camera arm *A* consists of a horizontal section of tubing brazed into the the lower part of the main valve at one end and terminating in a flange *P* at the other end. Bolted to *P* is flange *O*, which carries all the internal and external parts of the camera assembly. Motion transporting the camera into position in the X-ray beam is transmitted from outside the tube by means of a magnetic coupling (N, U). In FIGURE 6, the camera is shown at L in a retracted position, withdrawn from the X-ray beam. In this position, the main valve may be opened or closed without disturbing the camera L or its carriage K. The carriage is mounted on four wheels M that ride in tracks mounted in a cylinder (not shown) attached to Flange O.

Passing through flange O is a rectangular tubing T that is closed at the right-hand end and is external to the camera arm. T carries a plunger Q with a permanent magnet U attached to it. Carriage K straddles the tubing and also has a permanent magnet N with its pole pieces adjacent to those of U. When the plunger, Q, is moved by means of a knob and rack and pinion, the magnetic coupling between U and N is sufficient to carry the camera carriage with it. Mounted on the roof of the chamber is an adjustable stop S, which can be set so that each time the camera is moved into the X-ray beam axis it will center at the same position because of rod R meeting S.

A novel feature of the camera arm is a red safelight mounted inside the chamber. This facilitates the insertion of the camera through the loading port into the camera carriage holder in the dark.

FIGURE 2 shows an exploded view of the camera (L in FIGURE 6). The tissue to be photographed is floated on the emulsion side of the photographic

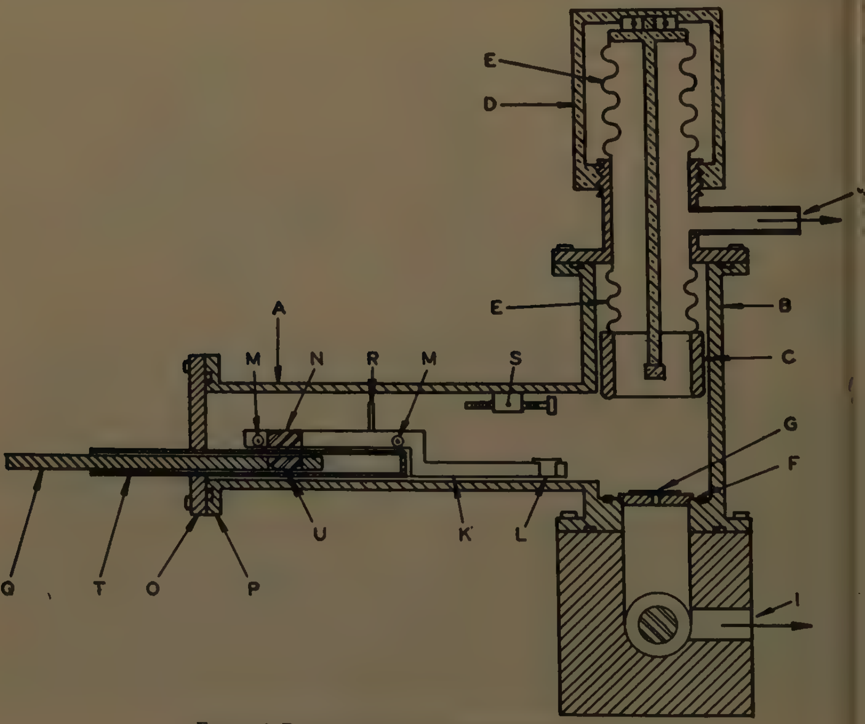


FIGURE 6. Drawing of main valve (B) and camera arm (A).

film, and the film is placed in the circular recess in the bottom of the camera body with the tissue facing down. A retaining ring presses the film against the camera when the cap is screwed onto the body. The whole assembly is dropped into place in the camera carriage through the camera arm port, and all handling of film is done under a red or yellow safelight.

(b) *Vacuum system.* Since biological specimens may have substances of unknown and high vapor pressure, the vacuum equipment must be capable of high speed and must be reliable to avoid long periods of pumping and breakdowns.

FIGURE 7 gives a schematic diagram of the complete vacuum system. The main backing pump *A* is mechanical, with a free air capacity of 1.7 liters/second and an ultimate vacuum of 50 microns of Hg. It provides an intermediate vacuum for molecular flow in the diffusion pump. Flange joint *B* connects backing pump *A* to a diffusion pump *C*. The diffusion pump is a metal, three-stage, fractionating, oil type having a speed rating of 250/liters/second at 3×10^{-5} mm. of Hg. Baffle plates installed to prevent oil scattering into the system reduce the speed to approximately 150 liters/second. Ultimate rated vacuum of this unit is 4×10^{-7} mm. of Hg. at 25° C.

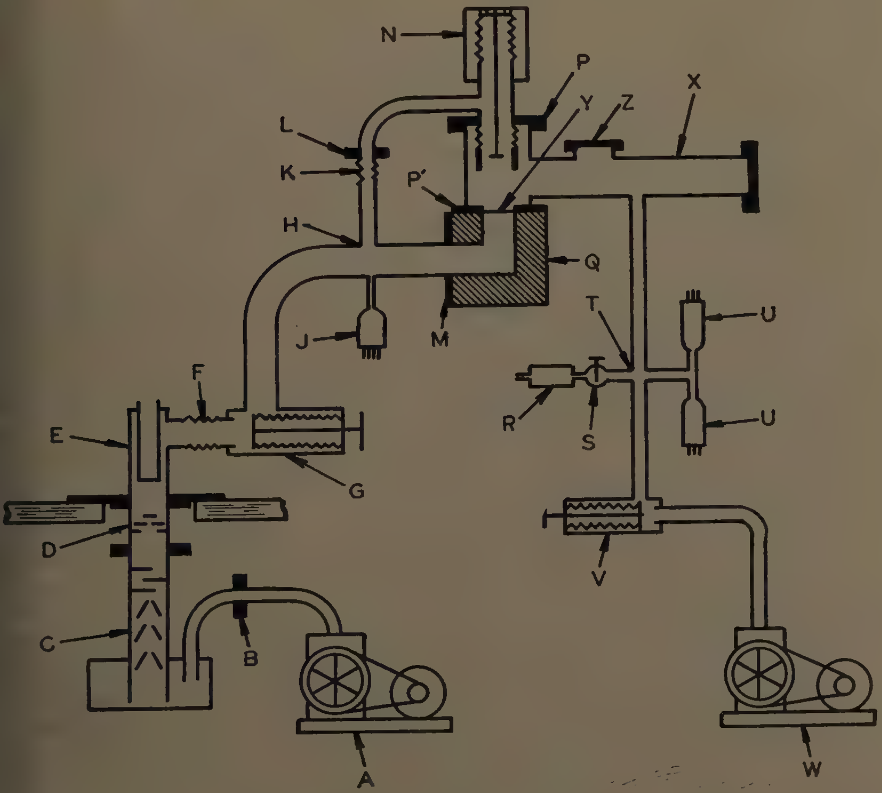


FIGURE 7. High vacuum system.

A water-cooled oil baffle (D) is a safeguard against oil films contaminating th apparatus. It leads directly into a cold trap E. A "T" section H connect the right angle valve G to the tube body Q through a flange joint M.

The second arm of the "T" section connects to the upper main valve assembly N through a syphon bellows K and a flange joint L. At the junctio of the "T" section, there is attached an ionization gauge tube J for measurin the pressure in the system. Its range is from 10^{-3} mm. to approximately 10^{-} mm. of Hg. Ionization of the gas present is accomplished by a constant-intensity stream of electrons, and the resulting positive ion current is a measur of the pressure. The upper main valve assembly N connects to the camer arm assembly with a flange joint P. The camera arm assembly X is attache to the tube body Q with a similar flange joint P'. When the main valve i closed, the X-ray tube is evacuated on both sides of window Y and the camer assembly X is isolated. When the main valve is open (as in FIGURE 7), hig vacuum pumping of the camera arm also takes place, in addition to that of th tube body.

To insert the camera through the port Z, one must admit air into the camer arm X. For this reason, the main valve is closed during loading, so that th tube proper will be kept evacuated.

An auxiliary rough pumping system W is provided to remove most of th air in the camera arm before valve N is opened. W is a mechanical pum having a free air capacity of 35 liters/second and an ultimate vacuum o 0.1/micron of Hg. A metal tube from pump W leads to a ⅝-in. right-angl vacuum valve V, which permits the pump to be isolated from the rest of th system when high vacuum pumping of X is being carried on. The exit fro valve V connects to one arm of a four-way cross joint, T. The second ar of joint T goes to a small needle-type air admission valve S, then to a dryin tube R containing Drierite. Air is admitted through R and S, so that the po Z can be opened for insertion and removal of samples. The third arm of carries two thermocouple vacuum gauges U, which indicate pressures i the range of 100 microns of Hg. One gauge reads pressure directly, whil the other is connected to a warning light that operates whenever the pressur in the camera arm is too high to permit opening the main valve N. Th fourth arm of T is connected directly to camera arm X. With this syste it is possible to insert the specimen and pump down to a pressure of $5 \times 10^{-}$ mm. of Hg in about four minutes.

(c) *Power supply.* The electronic operation of the X-ray tube requires low-voltage alternating current supply for heating the tungsten filament an a high-voltage direct-current supply to maintain the target at the desire potential (FIGURE 8).

The tungsten filament requires about 6 volts and 9 amperes, or 54 watt to reach a sufficiently high temperature to provide the 34-milliampere targe current desired. To supply the low voltage and high current, a 12-to-1 ste down transformer is controlled by a variable auto transformer connected t the 220 volt alternating-current lines. An ammeter in the secondary of th step-down transformer permits resetting to specific conditions. Various r lays are included for automatic operation.

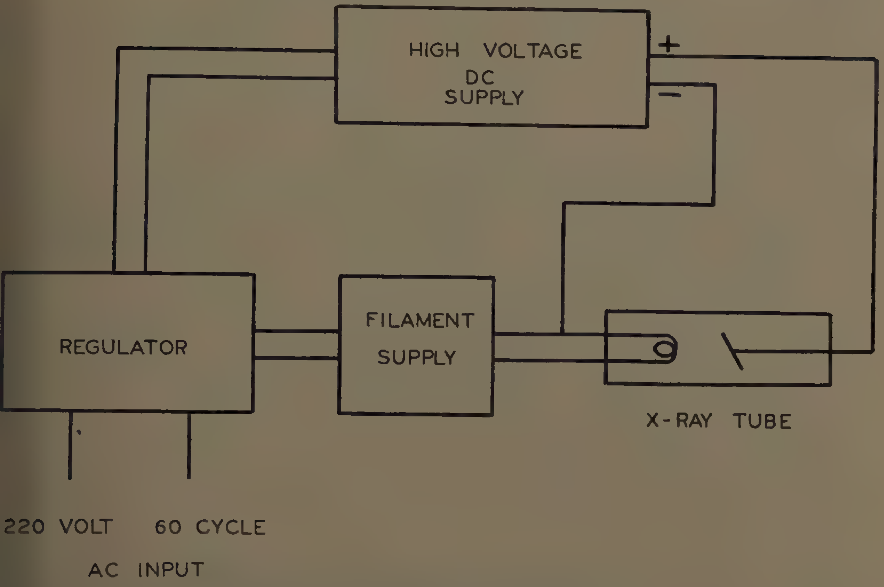


FIGURE 8. Schematic diagram of power supply.

The X-ray tube is usually operated at 3000 volts and 34 milliamperes, but 2000 volts and 24 amperes, and 1500 volts and 16 milliamperes have been used. The direct-current high-voltage unit will deliver up to 8000 volts at 300 milliamperes. The power supply operates from 120 to 220 volts, single phase, alternating current, and contains a step-up transformer, controlled by variable auto transformers to provide the high-voltage alternating current that is converted to direct current by a voltage doubler-type rectifier having sufficient filtering to give a maximum of 1 per cent ripple. Regulation at maximum voltage from no load to full load is 15 per cent. The negative terminal of the supply is grounded, and output to the tube is through a shielded coaxial cable. Dual-range meters indicate current and voltage, and an adjustable overload relay makes the supply inoperative if a predetermined current is exceeded.

Line-voltage regulation is provided for both filament and high voltage supplies, so that this parameter will be constant under varying load conditions. A simplified block diagram of the power-supply installation is given in FIGURE 8.

(d) *Control equipment.* Several control devices have been incorporated in the apparatus. The ionization gauge circuit is so constructed that if the tube pressure rises sufficiently to throw the meter off scale, a relay opens and disconnects the ionization gauge and removes the power input to the X-ray filament supply. When this happens, an interlock in the high voltage supply is automatically opened and power is shut off. During operation of the tube, power from the primary side of the filament circuit is fed through a time-delay relay to the exposure timer and operates a holding relay to keep the interlock closed.

In addition, the foregoing circuits act to prevent application of the high volt-

age before the tube filament has reached operating temperature. A synchro
nous timer allows any exposure to be made, from a few seconds to one hou
to the nearest second. Initiation of an exposure is made by a switch on th
timer.

To prevent accidental damage to the camera by closing the main valve o
it when the camera carriage is positioned in the main tube, a control circu
has been designed which, by the aid of limit switches on the camera carriag
and the main valve, rings a bell when an attempt to close the valve is mad
Switches are also connected to a system of pilot lights on a panel to inform th
operator where the movable elements are at all times.

The complete X-ray unit is shown in FIGURE 9.

(e) *Processing*. Gaevert Lippman emulsion has been used on occasion
but we prefer the new 10-micron-thick 649-0 Eastman Kodak emulsion on
35-mm.-film base. This emulsion gives a linear response with densities up t
at least 1 when plotted against intensity.

Most of our microradiographs are taken at a 2- to 4-minute exposure wit
X rays produced by 3 kv. and 34 m.a. Filament current is 8.9 amperes. Tw
kv., 1.5 kv., and 1 kv. have been used for greater contrast in some preparation

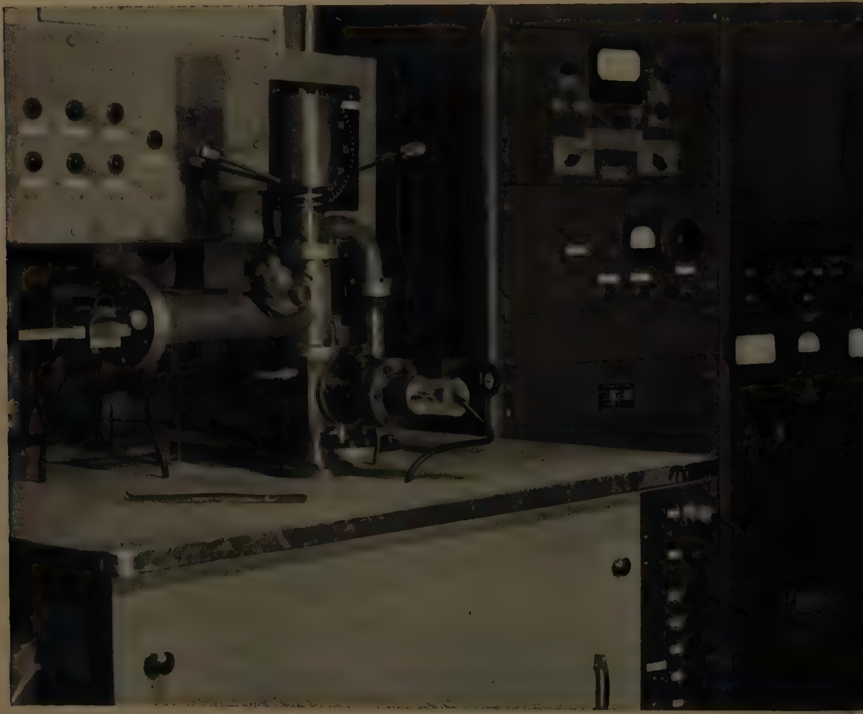


FIGURE 9. Photograph of X-ray unit. To right is power supply, to the left of it is the electronics rack wi
voltage stabilizer and ionization gauge and thermocouple controls. Tube rests on a cabinet with an overlyi
control light panel with timer. Pumps in cabinet.

The vacuum is about 8×10^{-5} mm. Hg. The microradiograph is developed n straight D-19 solution for 7 to 10 minutes at 19° C.

4. *Tissue Mounting*

Engstrom mounted the specimens as illustrated in FIGURE 4, 1, and we origi-ally used a slightly different method (FIGURE 4, 2). More recently, we have loated a paraffin-embedded section directly on emulsion and deparaffinized t by gently dropping xylol on the preparation. After drying, the whole prepa-ation is dipped in xylol to remove all the paraffin from the emulsion. The issue can be removed from the emulsion after exposure and stained.[18]

5. *Microdensitometer*

To measure the density (or transmission) of small areas of a few microns iameter in the microradiograph, it is necessary to magnify these areas by ome means. We have magnified the microradiograph by visible light micro-copy and have measured the transmission of light by a densitometer placed n the image plane to determine the optical density of the microradiographic reas (FIGURES 10 and 11). The microscope unit is a modified Leitz Biological anphot system. Illumination is furnished by a 100-watt zirconium arc

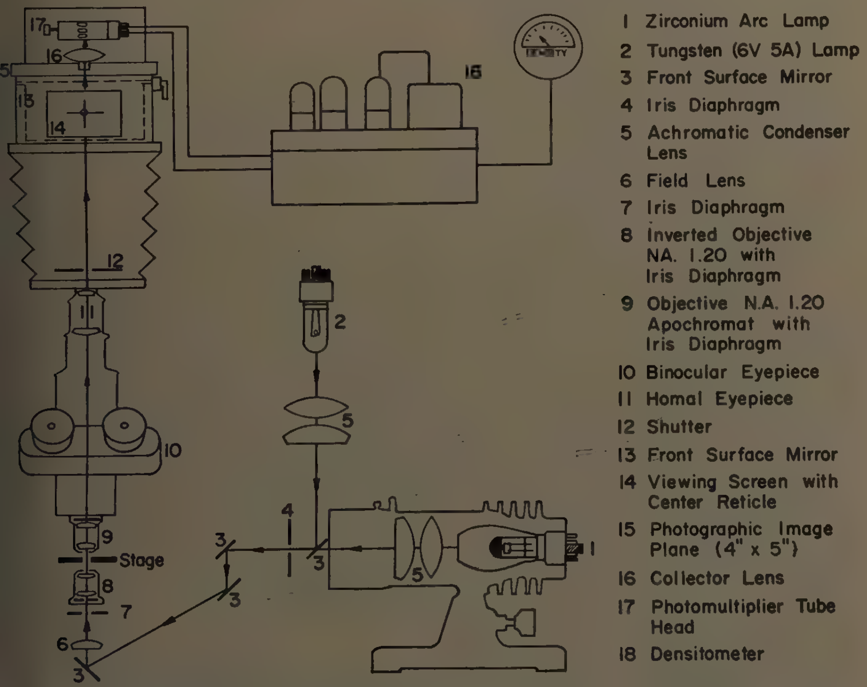


FIGURE. 10. Schematic diagram of microdensitometer. Recent modifications include substage objective lower N.A. (0.50), objectives without diaphragms, iris diaphragms between eyepiece (11) and shutter (12) and photographic image plane (15).

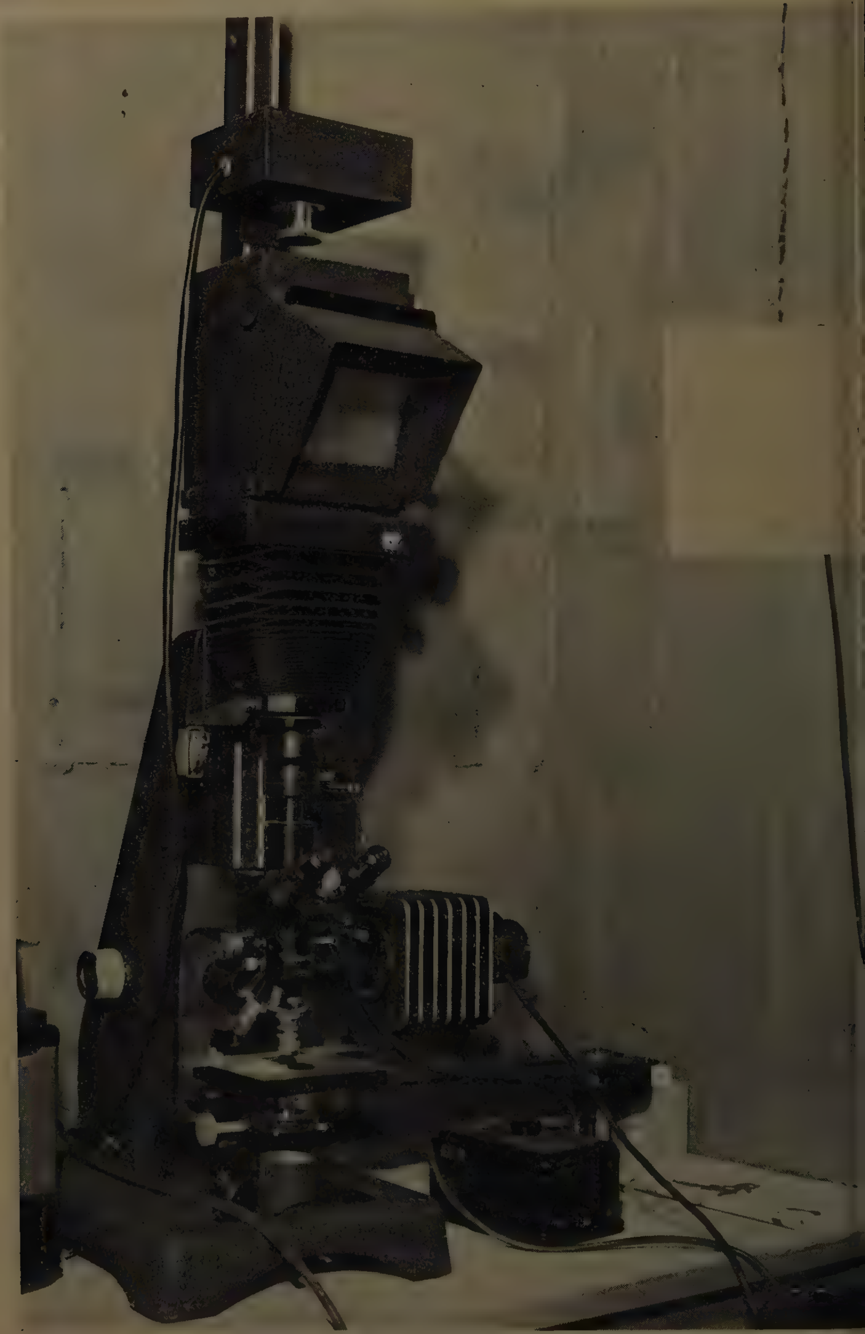


FIGURE 11. Photograph of microdensitometer.

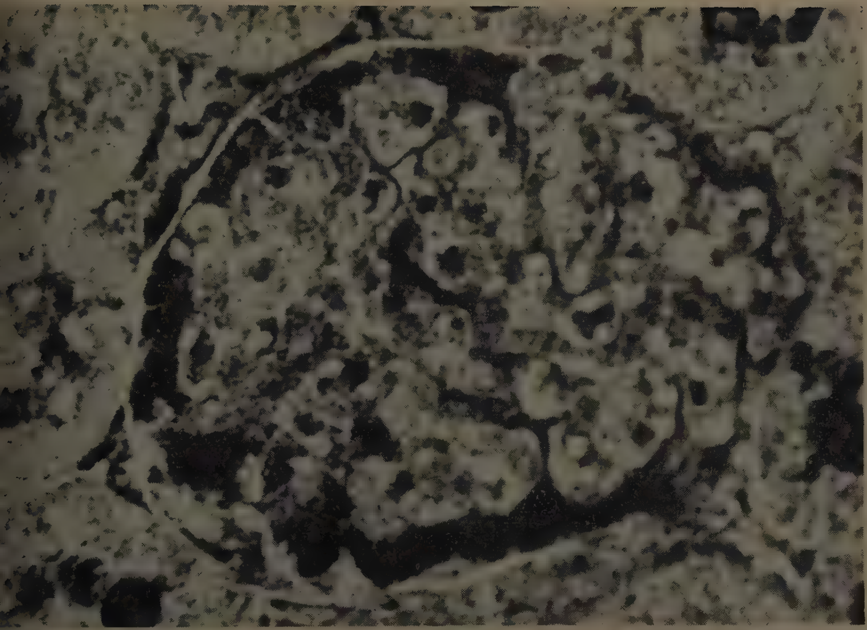


FIGURE 12. Microradiograph rat kidney. Glomerulus with considerable mass. Convoluted tubules have uch cytoplasmic and nuclear mass. ×1000.

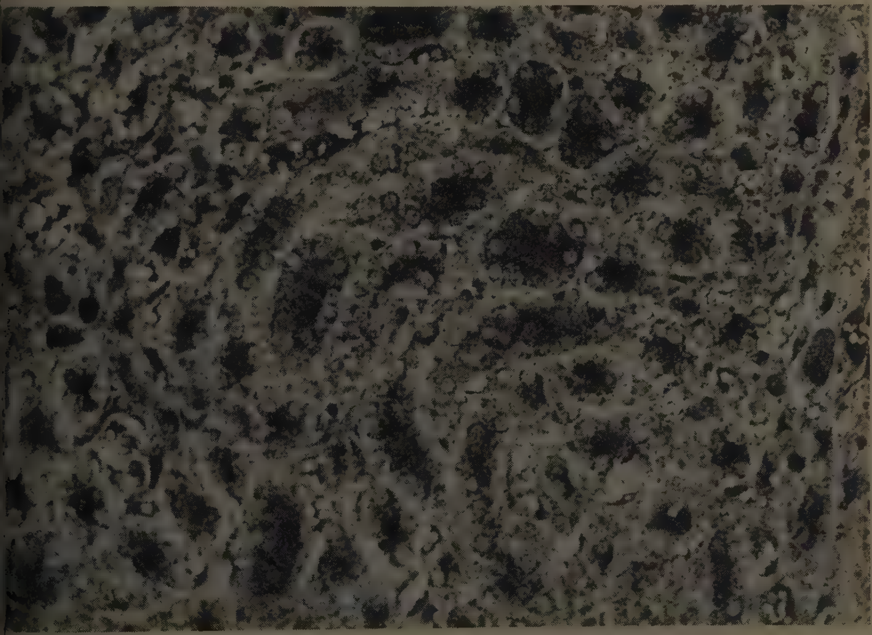


FIGURE 13. Same tissue section as FIGURE 12. Medullary region with proportionately more nuclear than toplasmic mass in collecting tubules when compared with convoluted tubules. ×250.

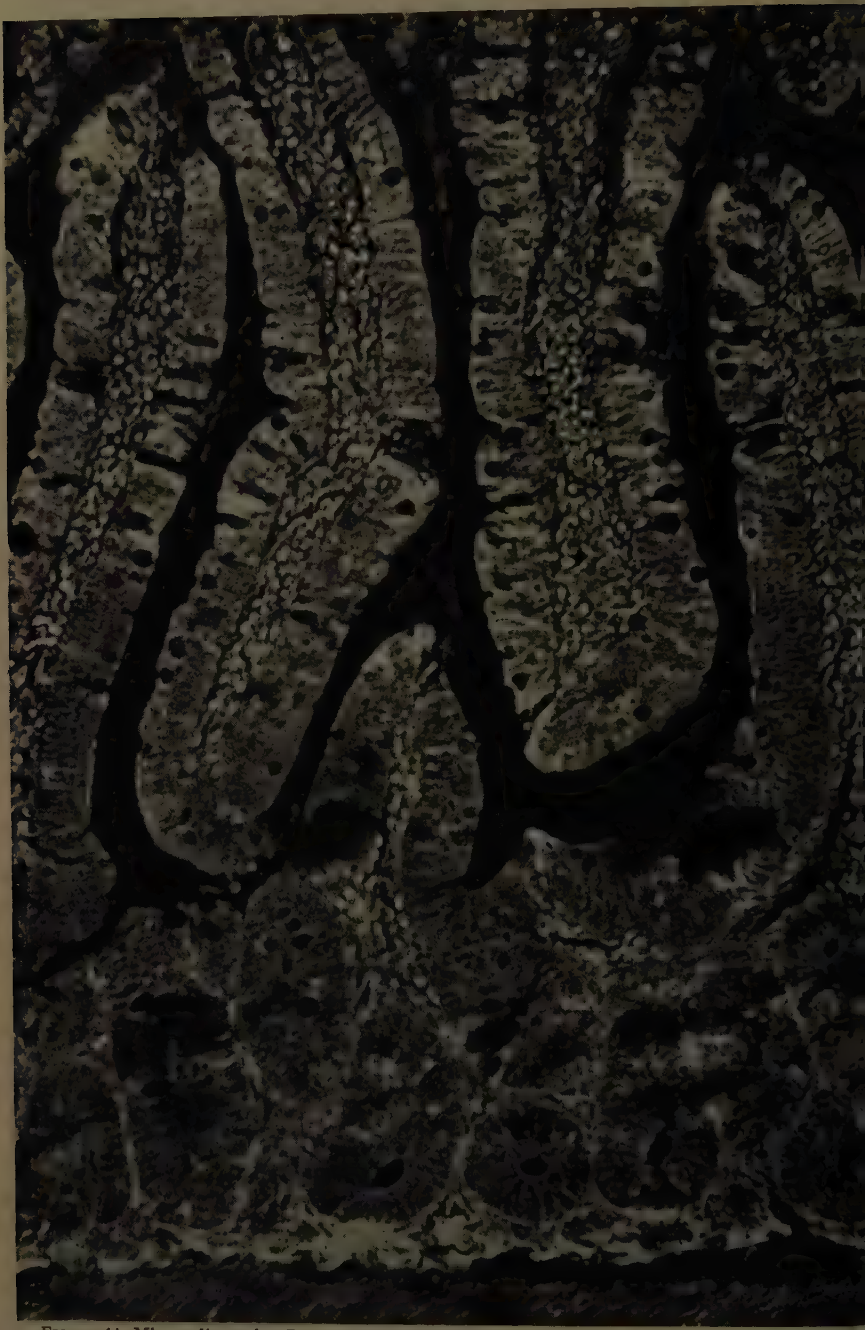


FIGURE 14. Microradiograph. Rat small intestine. Goblet cells apparent by the low mass concentrat
in goblet. A cuticular border with a slightly greater mass than the adjacent cytoplasm is seen in some ar
×200.

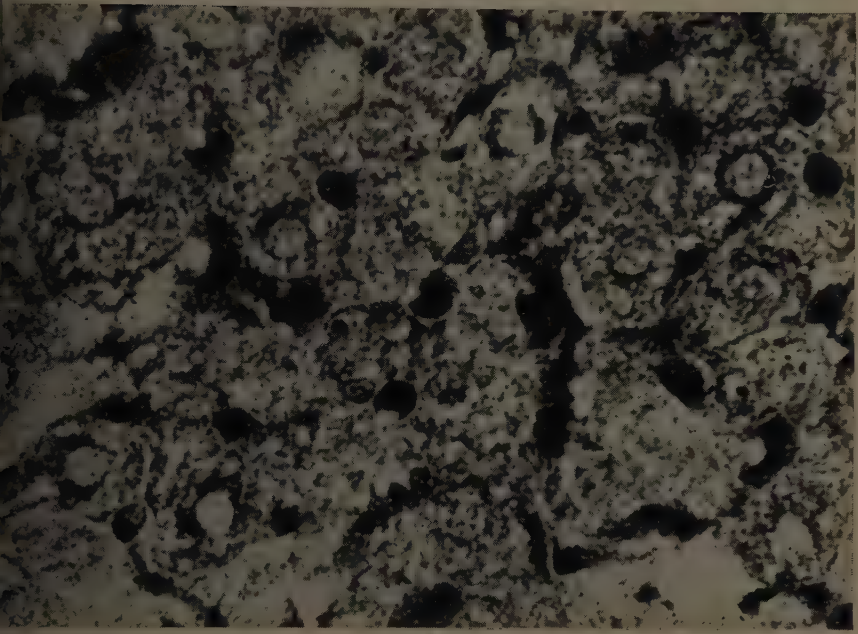


FIGURE 15. Microradiograph. Rat liver. Considerable nuclear and cytoplasmic mass. ×750

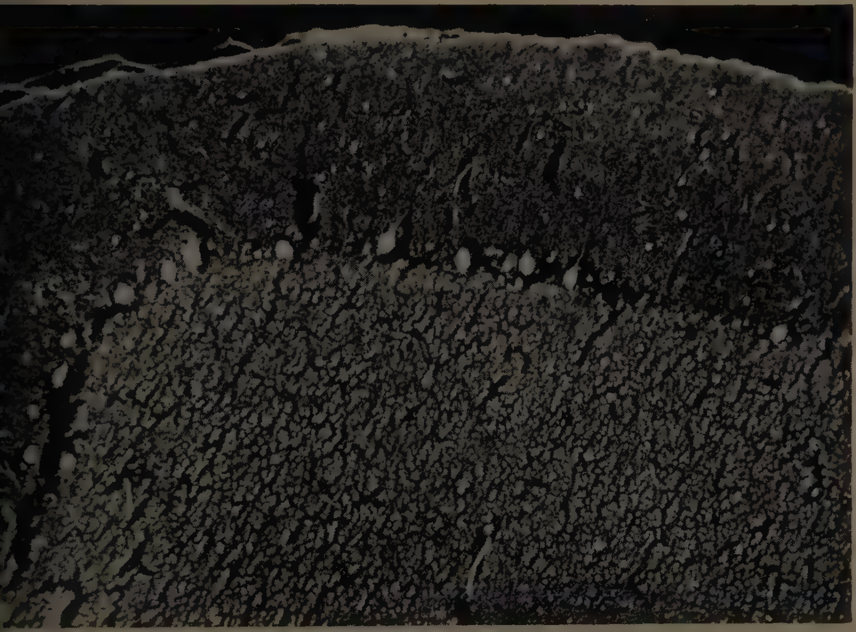
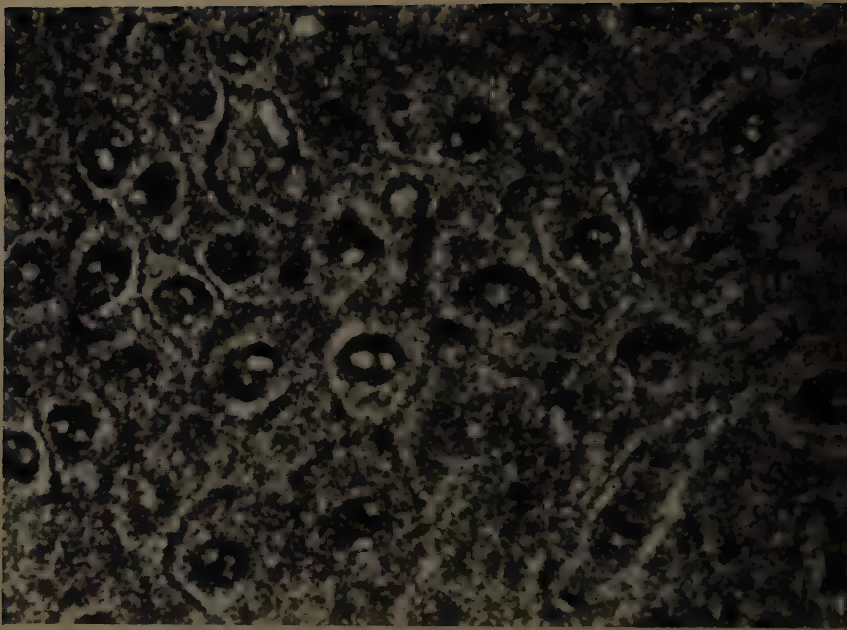


FIGURE 16. Microradiograph. Rat cerebellum. Pia mater has considerable mass. The stratum cinereum s much less mass concentration than stratum granulosum. Heavy concentration of mass in the Purkinje ls and their dendrites, in granular cells and in small cells in the stratum cinereum. ×50.



· FIGURE 17. Microradiograph. Human buccal mucosa. Little or no nuclear mass except where nucleolu
nuclear chromatin is present. Both show high mass concentration. Considerable mass in cytoplasm in
region. Intercellular bridges discernible in microradiograph and have more mass than intercellular "spa
×1000.

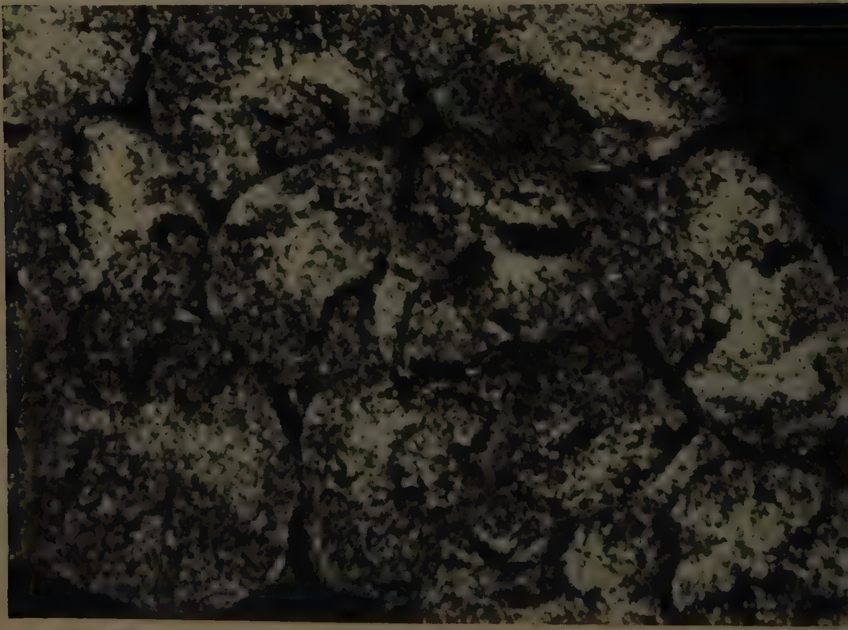


FIGURE 18. Microradiograph. Normal rat pancreas. Greatest mass in apical portions of cells where zy
gen granules present. ×1000.

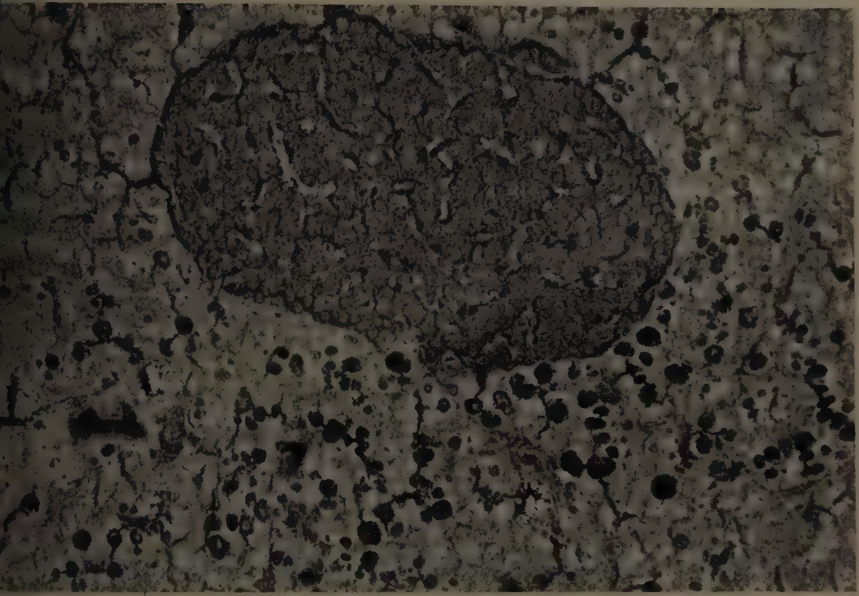


FIGURE 19. Microradiograph. Rat pancreas. Early ethionine effect (vacuolization and loss of cytoplasmic ass). Islet unaffected and has less mass than acinar cells. ×150.

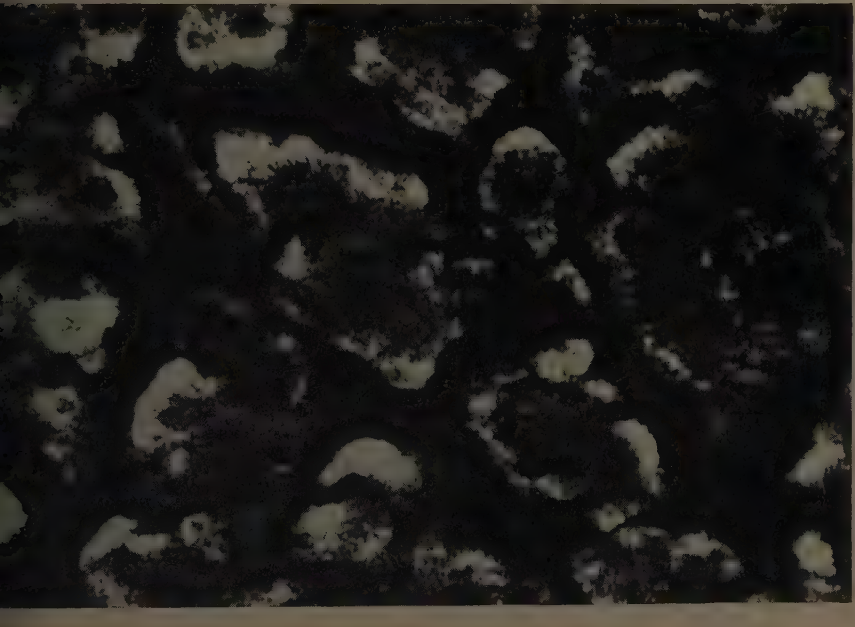


FIGURE 20. Microradiograph. Rat pancreas. Regeneration of acinar cells subsequent to ethionine. Heavy ss concentration in basal portion of cells. Compare with normal FIGURE 18, where most mass is apical. ×900.

source (FIGURE 10, 1). The densitometer is a Macbeth-Ansco Color Den tometer with a modification of the photomultiplier head (FIGURE 10, 17).

A feature incorporated in the optical system is the use of an objective (FI URE 10, 8), numeric aperture 0.5, and an ocular (FIGURE 10, 6) acting as a co denser to illuminate a field only slightly larger than that of the object measure This was suggested by Naora[19] as a means of decreasing Schwarzchil Viliger effects. With diaphragms and the demagnification of the ocular-o jective condenser system, we can illuminate areas as small as 10 microns.

A tungsten ribbon lamp (FIGURE 10, 2) may be used to illuminate the who microscopic field for visual scanning through binocular eyepieces (FIGURE 1 10) or for photomicrography (FIGURE 10, 14 and 15). A front surface mirr (FIGURE 10, 3) permits either the zirconium arc or the tungsten lamp to used and the other to be excluded. The density or transmission may be re off the scale of the densitometer (FIGURE 10, 18). Or, a potentiometer-ty recorder (Leeds-Northrup Speedomax) attached to the densitometer is us

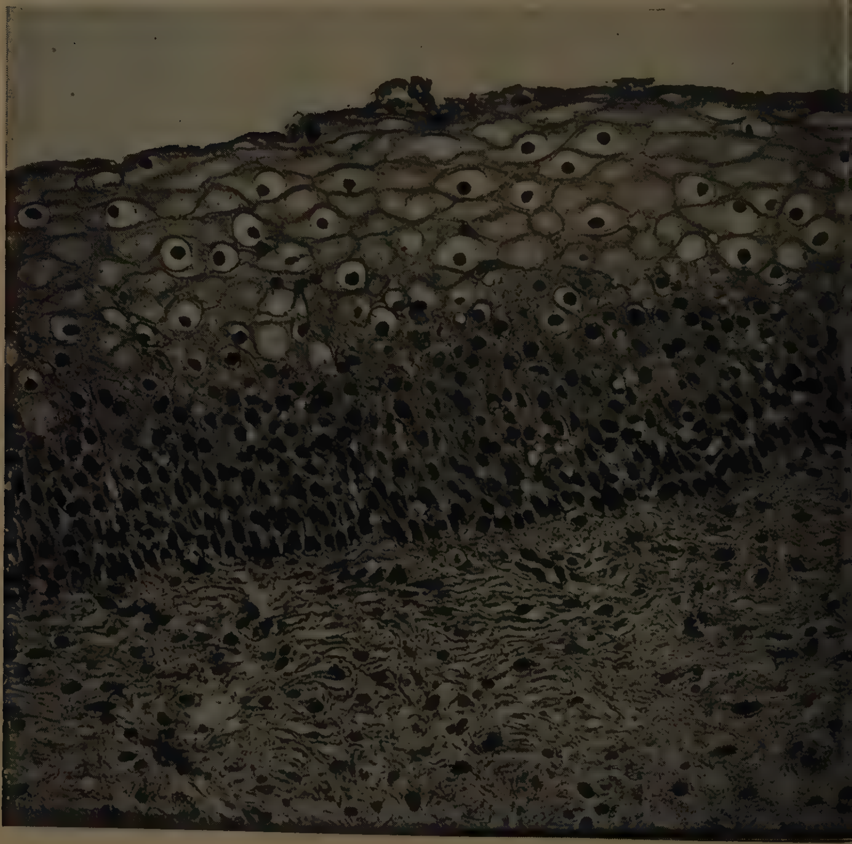


FIGURE 21. Histologic section. Normal human cervix. Hematoxylin and eosin. ×200.

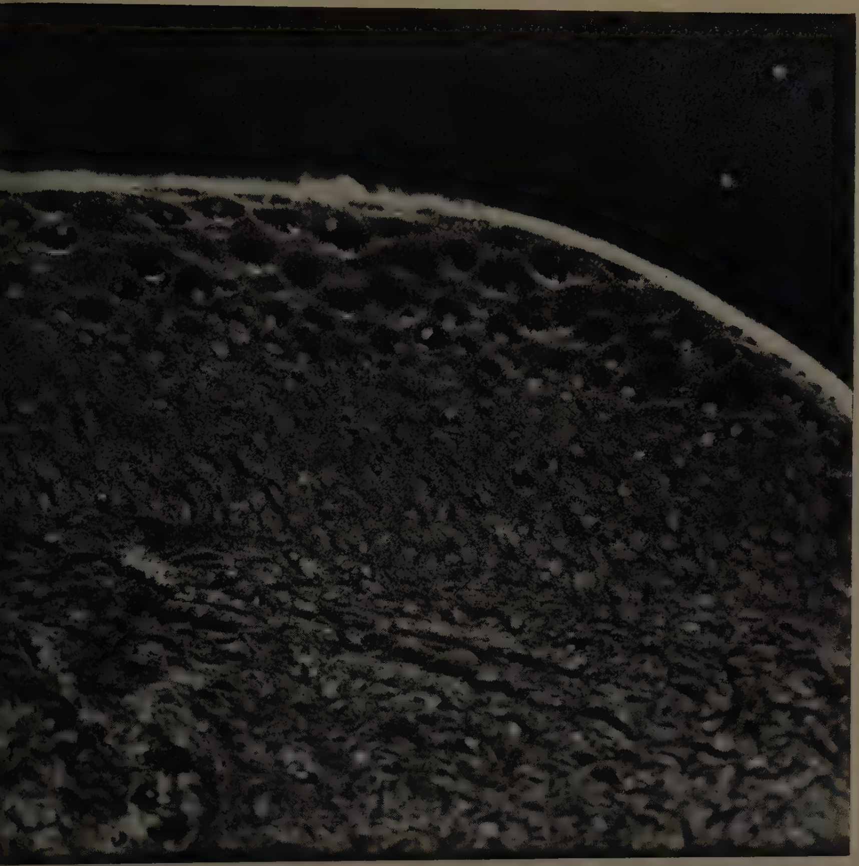


FIGURE 22. Microradiograph, section adjacent to that of FIGURE 21. High mass of keratinized zone, little no mass in vacuoles and intermediate mass of stratum spinosum and stratum germinativum. Nuclei have oderate mass. ×200.

record either the individual peaks of transmission or else linear traverses areas in the microradiograph. For movement across the stage required in e latter, we have used a motor-driven mechanical stage.

6. *Examination of Microradiograph*

All microradiographs have been studied visually by light microscopy and e distribution of mass in the tissues noted. Quantitative measurements are ing made.[18]

In all cases, either the histologic section that was used for X-ray absorpon, or the adjacent section has been stained with hematoxylin and eosin and ed for a control or guide in the interpretation of the microradiograph.

7. *Applications of the Mass Determination Technique to Biologic Objects*

Literature. Since the publication of Engstrom and Lindstrom's origi article,[1] a wide variety of tissues and cells have been examined. Centr nervous system cells have received considerable attention, especially fro Hydén and colleagues,[5, 12, 13, 15, 20, 21] who have examined sensory and mot nerve cells of the rabbit and chick embryo during growth, stimulation, a viral infections. Nurnberger *et al.*[4] have studied the relationship between t ultraviolet absorption and the mass of the ventral horn cells of the cat. Er strom and his colleagues have been particularly interested in bone growth a disease.[3, 22, 23] Dog gastric mucosa,[2] (*developing ova in the frog*), frog vertebr *Allium* root tip, and *Chironomus* muscle and chromosomes[2, 3] bacteria[24] a rat pancreas[25] have shown provocative microradiographic images.

Human skin,[1] kidney,[7] and thyroid[25] have been examined. As with so of the animal studies, these have been of a survey nature.

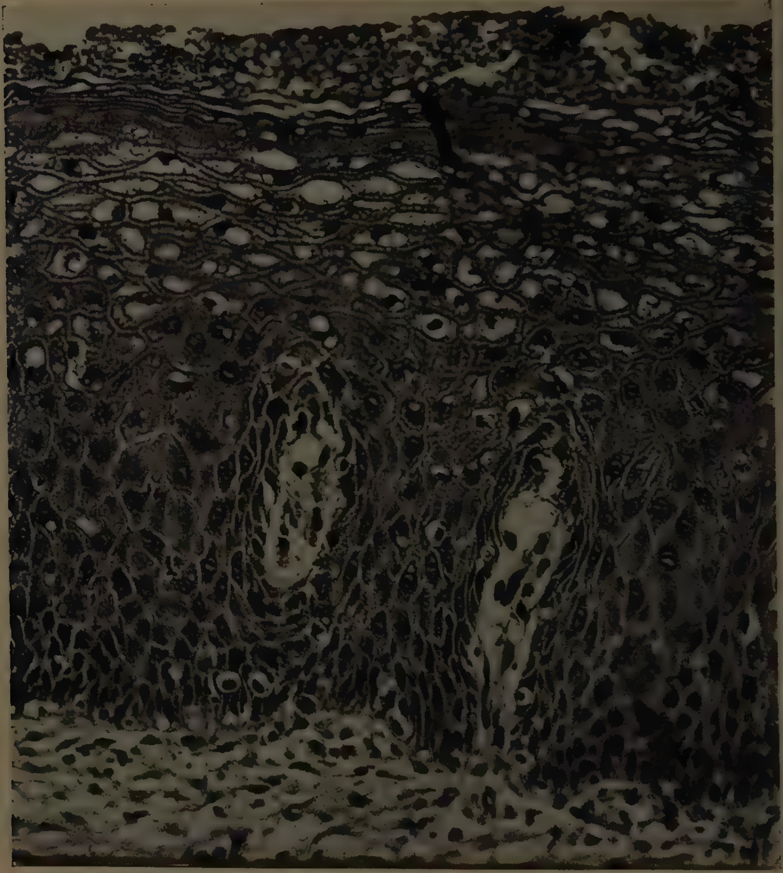


FIGURE 23. Histologic section. Human female cervix. Hematoxylin and eosin. ×300.

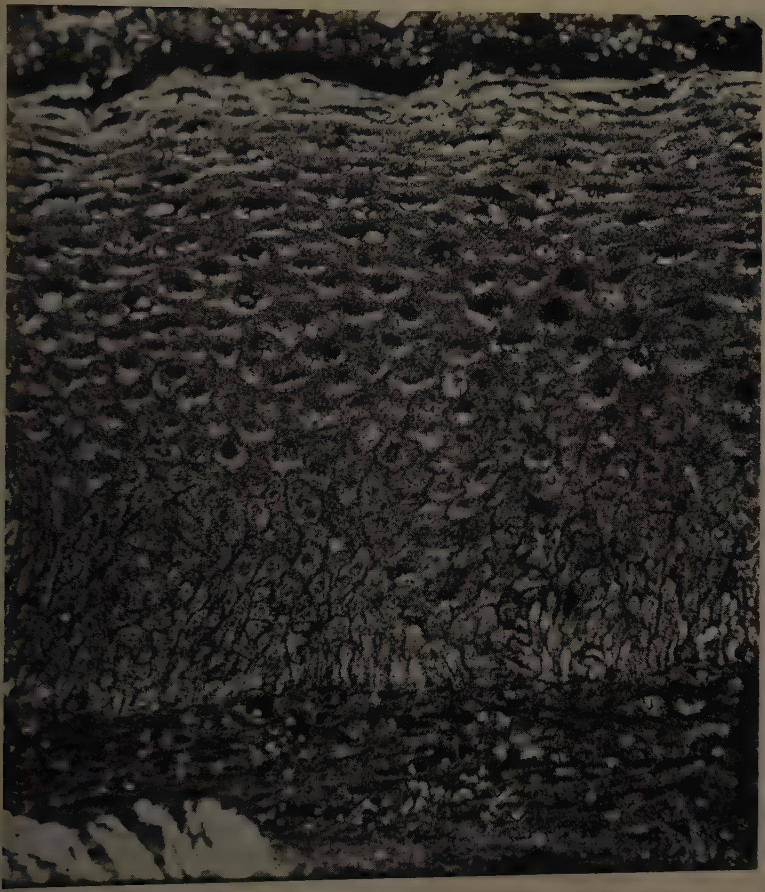


FIGURE 24. Microradiograph, section adjacent to that of FIGURE 23. Considerable cytoplasmic and nuclear ncentrations of mass in basal layer. Less in stratum spinosum except in nucleoli. Keratinized layer has avy concentration. Dark elliptical areas in outer zone are vacuolated areas with little or no mass. More ithelial mass concentration than connective tissue concentration. ×300.

Recently Lindstom and Moberger[9, 10] and Moberger[8] have studied vaginal nd cervical mucosa in the human female and have also examined the changes the vaginal mucosa of the mouse during carcinogenesis.

These authors noted that in the vaginal epithelium of mice, during carcinoenesis, the spinous layer of hyperplastic epithelium had a lower dry weight nass) than the normal epithelium. In the developed carcinomas, the periphal cell layers had a dry weight per unit area that was equal to or higher than at of the normal epithelium. Inner parts of cancer cords had a lower dry eight per unit area than had the spinous layer of the normal epidermis. hese results of X-ray analysis parallel the findings of Casperson and Santersson their study of the ultraviolet absorption in cancer[26] although, in some other findings, the two methods show a disparate relationship.[8] Previously, a si lar discrepancy in the two techniques had been pointed out in the thyroi

Moberger[8] found that in the regenerating, slightly hyperplastic epitheli of the mouse vagina, there was a significant decrease in the dry weight per u volume as compared with normal epithelium. In the precancerous hyp plasia, a similar decrease was found. In carcinoma variations, decreases dry weight were greater than in normal or hyperplastic epithelium. No s nificant difference in dry weight was observed between the peripheral cells cancer cords and the basal layer of normal epithelium.

Moberger's study of human epidermis comprised a nonneoplastic tubercul proliferating epidermis, a transition of a skin papilloma into epidermoid car noma, a precancerous papillomatous hyperplasia, a basal cell carcinoma, a

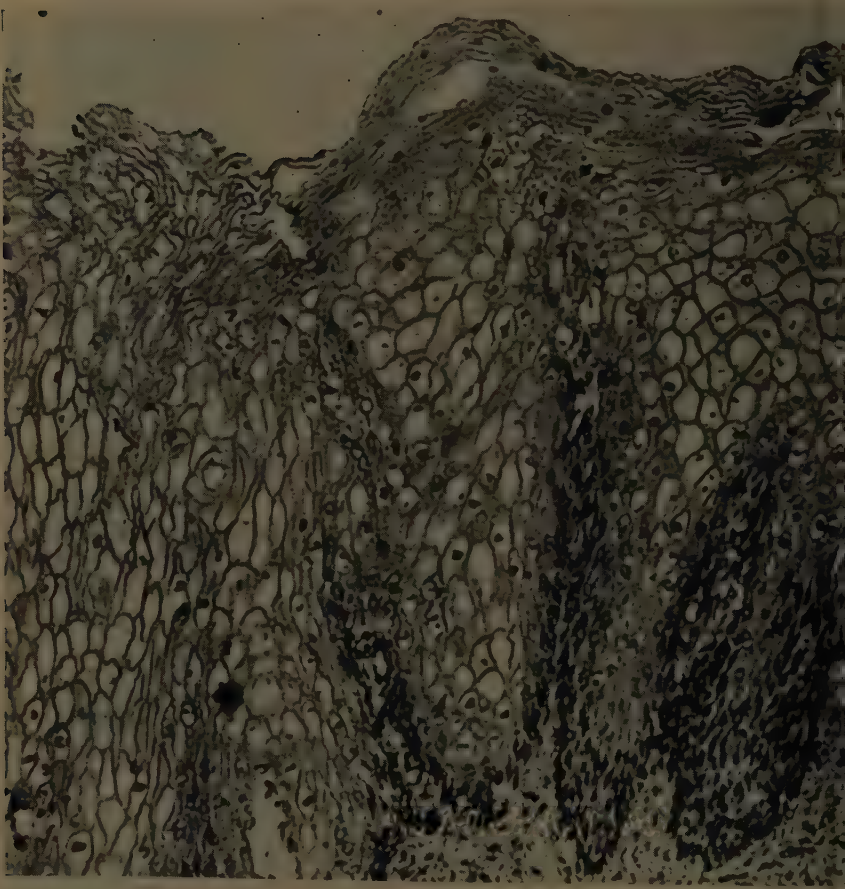


FIGURE 25. Histologic section. Human cervix uteri. Slight hyperplasia of basal layer. Hematoxylin eosin. ×300.

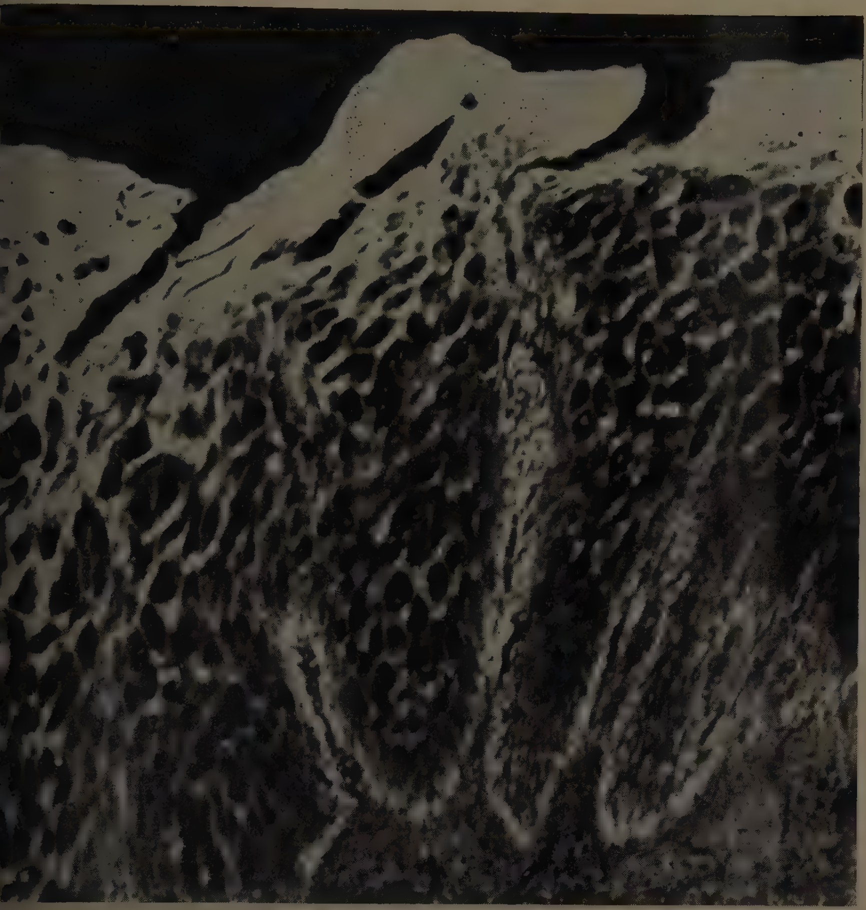


FIGURE 26. Microradiograph, section adjacent to that of FIGURE 25. Heavy mass concentration in keratin.
eavy concentration of basal layer. Skein of contiguous cytoplasms surrounding vacuoles, prominent nuclei
spinosum zone. ×300.

ur epidermoid carcinomas (one slowly growing vulva lesion and three rapidly
owing skin cancers).

In the tuberculous hyperplasia, a slight decrease of dry weight per unit vol-
ne as compared to normal epithelium was found. In the precancerous hyper-
asia, a slightly higher dry weight was found in the peripheral cords, but a
wer dry weight per unit volume in the inner cords of cells than in the corre-
onding normal epithelium.

In the basal cell carcinoma and in the peripheral cell layers of four epidermoid
rcinomas, the dry weight had the same value as in normal epithelium.

Where comparable lesions were studied, Moberger's results have been dupli-
ted in our studies.[18]

Rat tissues. A few of the organs of the rat that we have examined are pr sented for illustrative purposes and some general features are mentioned.

Connective tissue elements such as bone, cartilage, fibrous, collagenou muscular, and elastic tissues show considerable mass, as do many nerve fibe Red blood cells have much mass, and can be used for rough internal standar or for visual estimates of relative mass.

Epithelial tissues vary considerably from organ to organ. Stratified squ mous epithelium may show a heavy mass concentration in a proliferati basal layer, less concentration in the spinosum, and heavy concentration the keratin and keratinizing cells (figures 21 to 24). Intracellular bridges c be discerned in the microradiograph (figures 17, 27, and 28), and they ha more mass than intervening substance in the intercellular space. Occasionall tonofibrils can be made out in the cytoplasm when they differ in mass fro the cytoplasm.

There appears to be a difference between the relative nuclear and cytopla mic masses in the proximal convoluted tubules (FIGURE 12) as compared the collecting tubules (FIGURE 13) of the kidney.

Goblet cells have a lower mass in the gobule of mucous than in the surroun

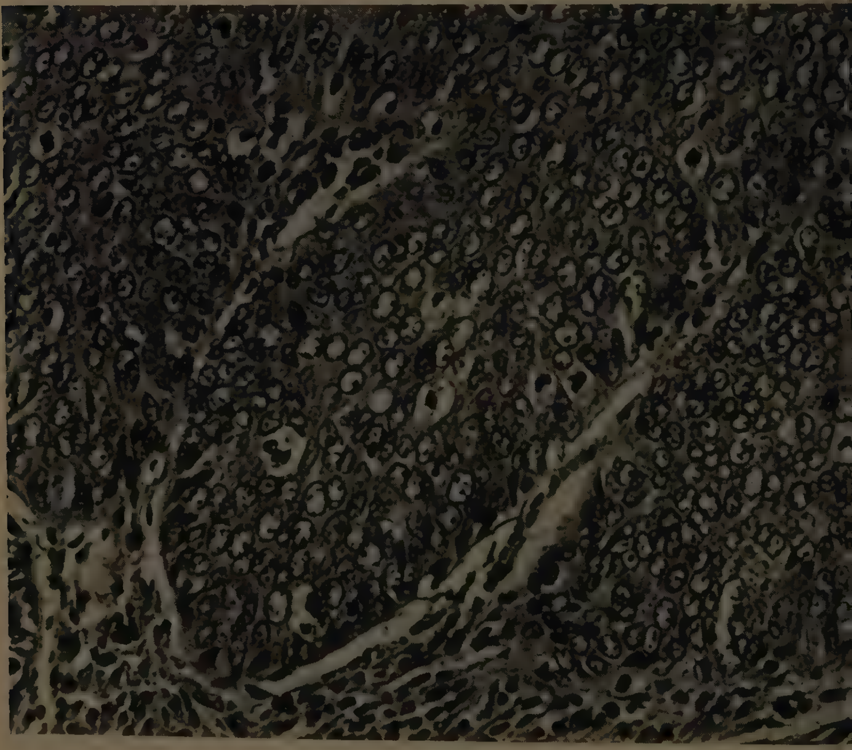


FIGURE 27. Histologic section. Atypical hyperplasia, human cervix uteri. Many mitotic figures pres Hematoxylin and eosin. ×300.

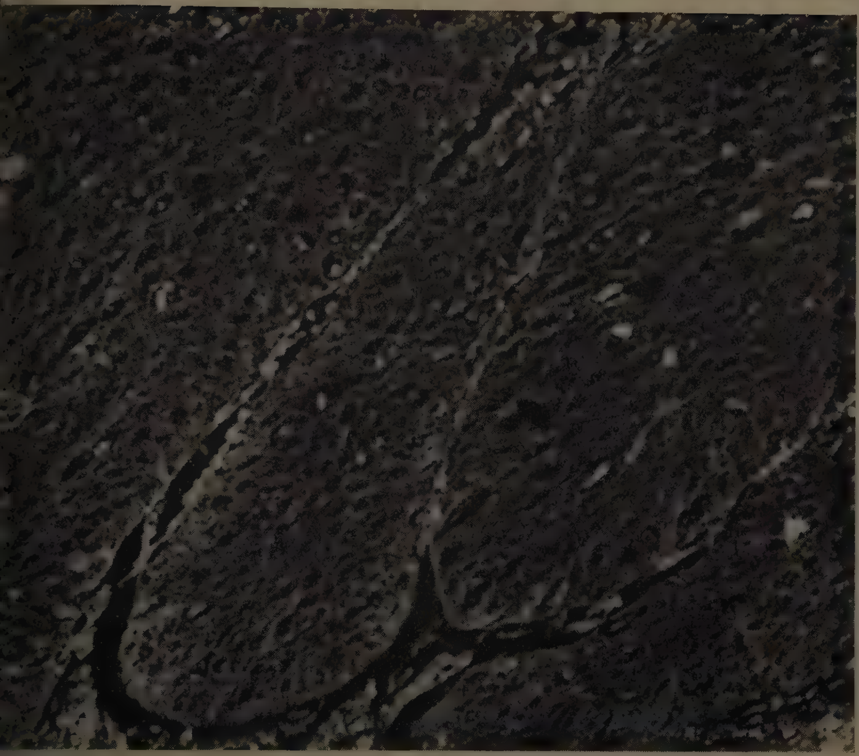
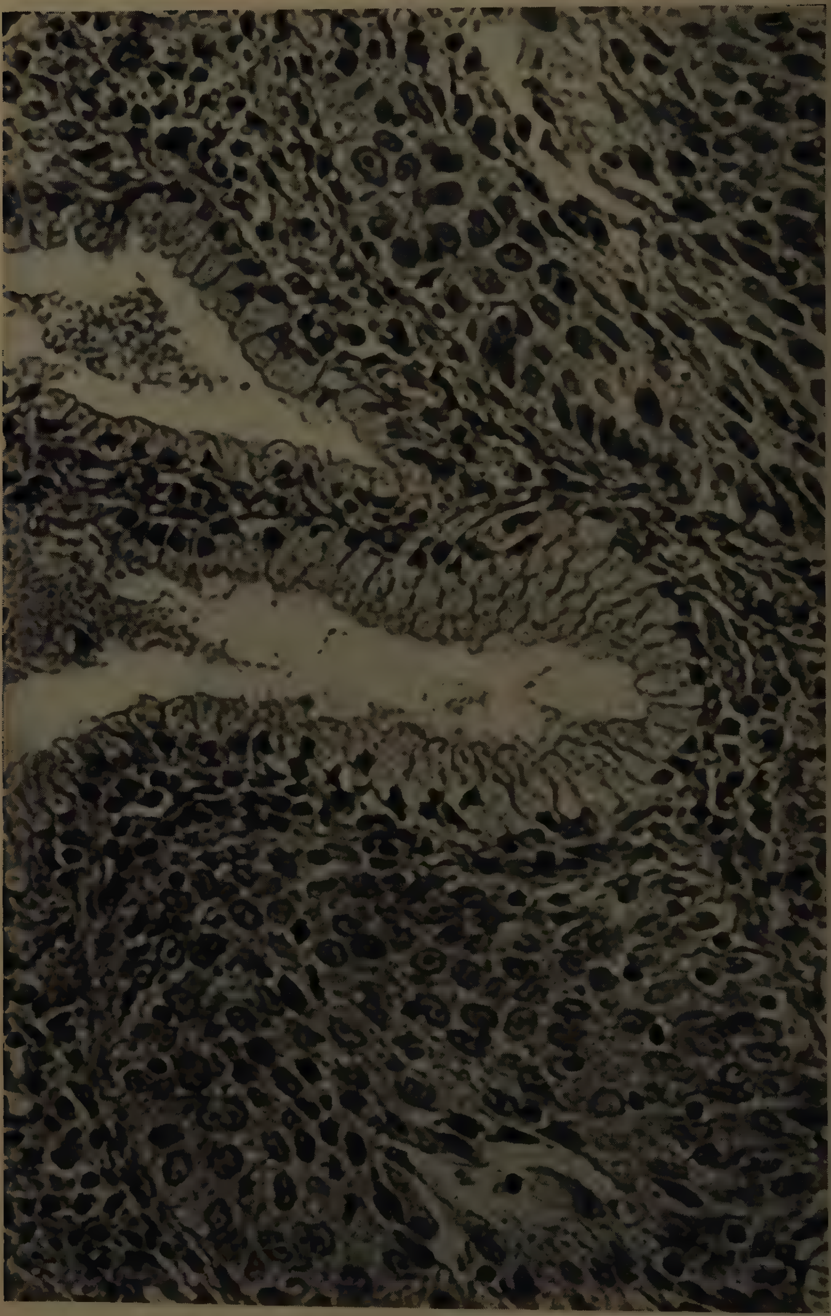


FIGURE 28. Microradiograph of section adjacent to that of FIGURE 27. Nuclei show relatively little mass
ept where mitotic figures or chromatin present. These have high mass. Cytoplasm has less mass than
romatin but more than nonchromatic areas of nucleus. ×300.

g cytoplasm (FIGURE 14). In the liver (FIGURE 15), nucleus and cytoplasm
ve about equal concentrations of mass. Pyramidal cells and the granular
lls of the cerebellum have heavy mass concentrations (FIGURE 16). Buccal
ucosa is representative of stratified squamous epithelium (FIGURE 17).
landular cells such as the pancreas may reveal secretory granules of high
ass concentration in the apex of the cell (FIGURE 18). Islet cells of the
ncreas have less mass than the acinar cells (FIGURE 19). Regenerating
ncreatic acinar cells have a high mass concentration at the base of the cell
IGURE 20), and the zymogen granules, with their high mass content, reappear
a later stage of regeneration.

Human. The normal human cervix uteri has been studied in comparison
th carcinoma *in situ* of the cervix.[18] In the normal cervix, keratin or kera-
izing cells have the highest concentration of mass (FIGURES 21 to 24). There
usually not a marked difference in the cytoplasmic mass concentration be-
een the basal and spinosum layers, although the former may have a higher
ount. In the zone where vacuolization is pronounced, the vacuole has a
gligible mass, whereas the surrounding cytoplasm has considerable concen-



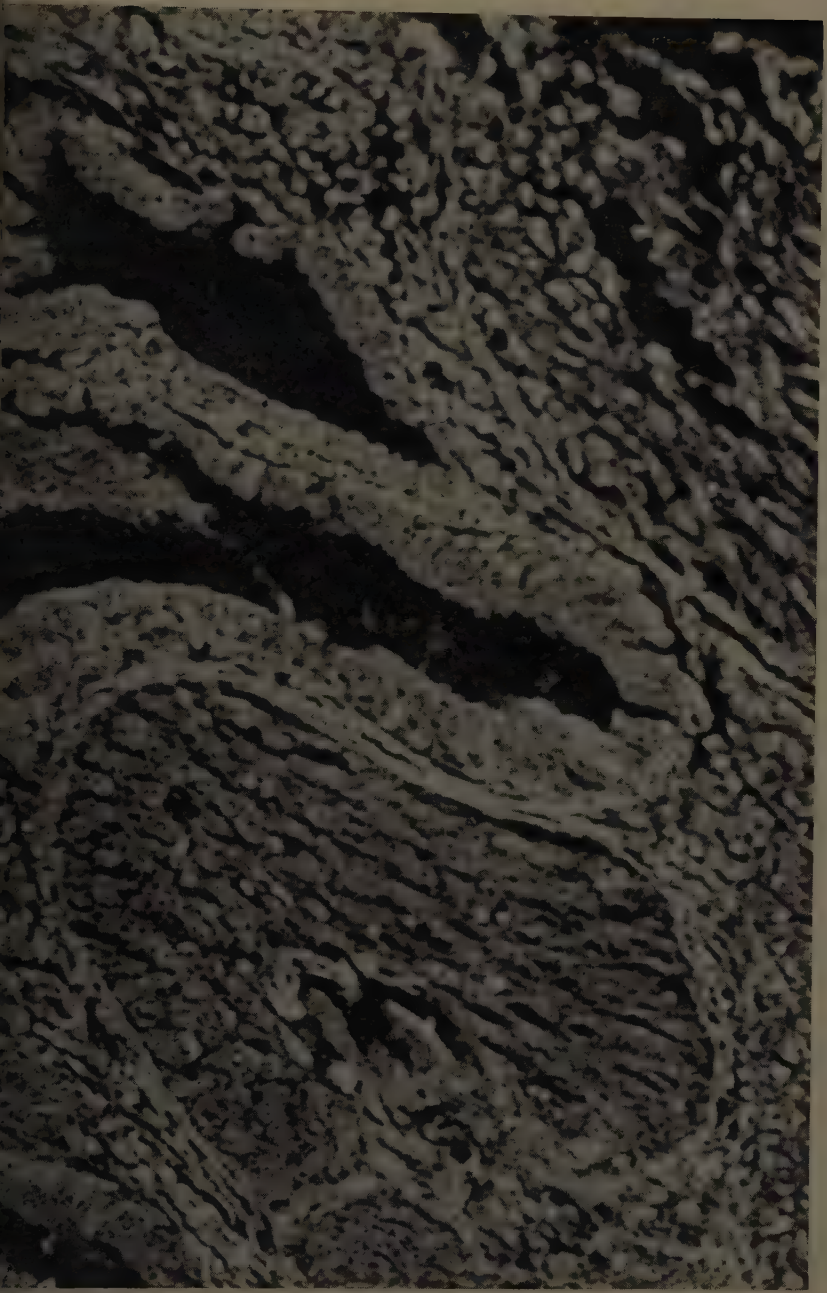


FIGURE 30. Microradiograph, section adjacent to that of FIGURE 29. Involved gland on left shows cytoplasm and nuclei with less mass concentration than normal mucous gland. Involved gland on right has cytoplasm somewhat similar to normal mucous gland but nuclei of involved gland show greater mass than mucous gland. ×300.

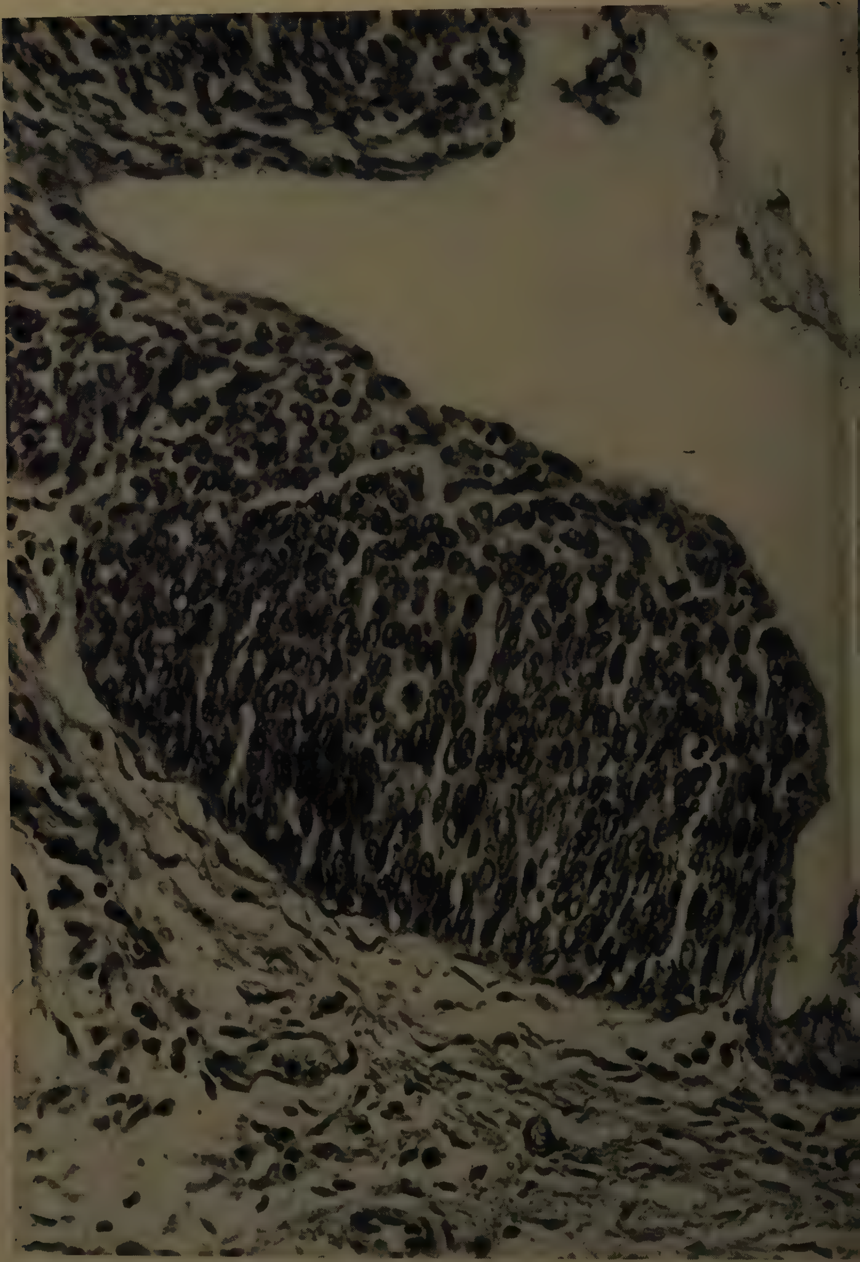


FIGURE 31. Histologic section. Human cervix uteri. Carcinoma *in situ* involving a cervical gland. Hen toxylin and eosin. ×300.

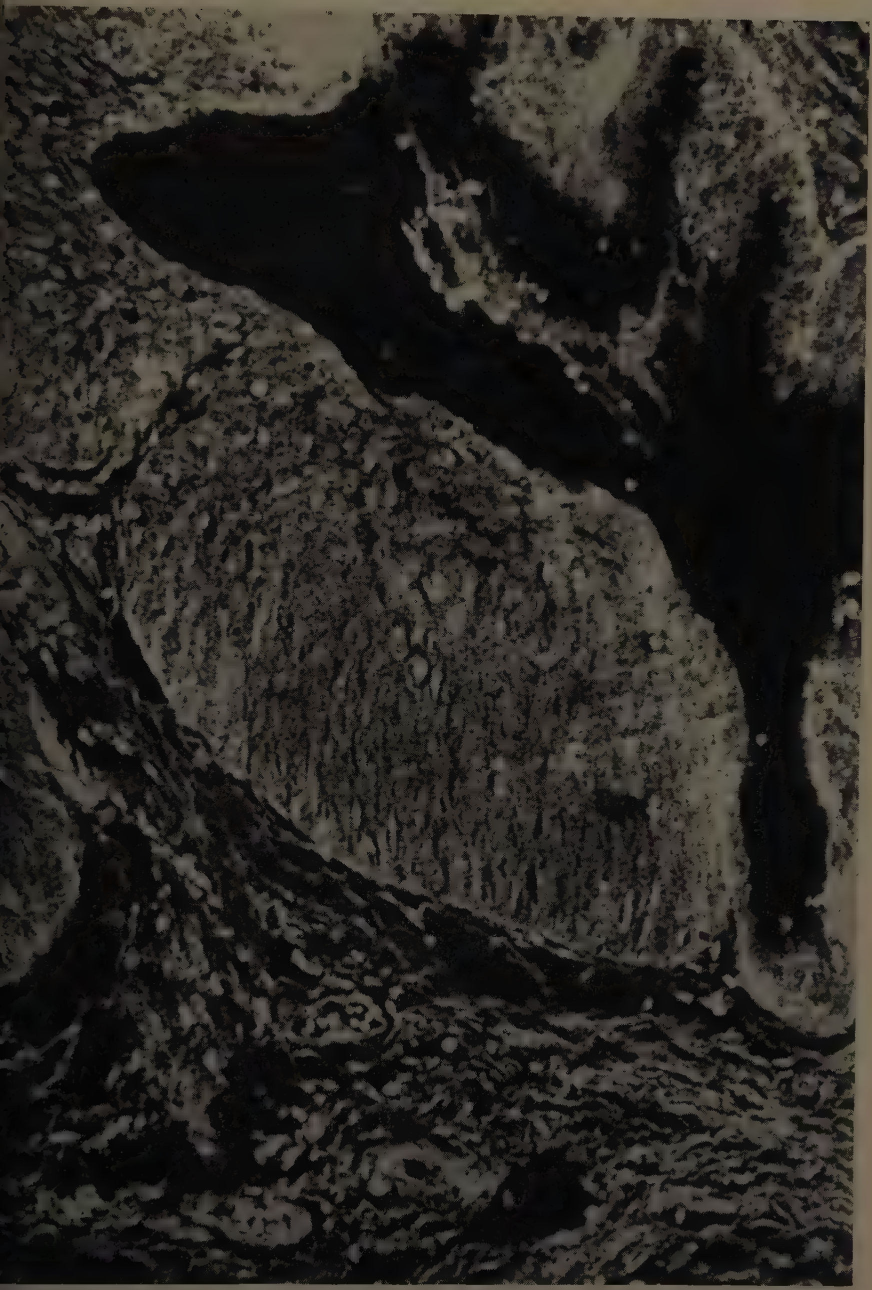


Figure 32. Microradiograph, section adjacent to Fig. 31. Variation in cytoplasmic and nuclear mass but th average lower mass concentrations than mucous of the gland. ×300.

tration. The nucleus is usually prominent in the lacuna of the vacuole becau
of its high mass. Whether the skein of increased cytoplasmic mass surroun
ing the vacuole is merely an expression of cytoplasmic condensation or t
beginning of intracellular keratinization has not been determined. The arr
of linear areas of heavy mass on the internal borders of the large vacuo
running parallel to the surface epithelium raises the possibility of the forma
fixative causing some intravacuolar or intracellular diffusion and concentrati
of mass in these regions.

Nuclei appear to have considerable concentration of mass in the basal laye
much less in the nonvacuolated spinosum zone (FIGURE 24) and more in t
vacuolated region (FIGURES 22, 24, and 26). Nucleoli have a great conce
tration of mass, and this is particularly noticeable in the spinosum region whe
the rest of the nucleus has relatively little mass (FIGURES 24 and 26).

Hyperplasia. In the cervical epithelium showing basal layer hyperplas
there is considerable increase over normal in the mass concentration (FIGUR
25 and 26). This presents a contrast to the spinosum zone when the vacuo
have relatively little mass and the skein of cytoplasm and intercellular spa
has a moderate amount. The keratinized layer has a very heavy concentr
tion.

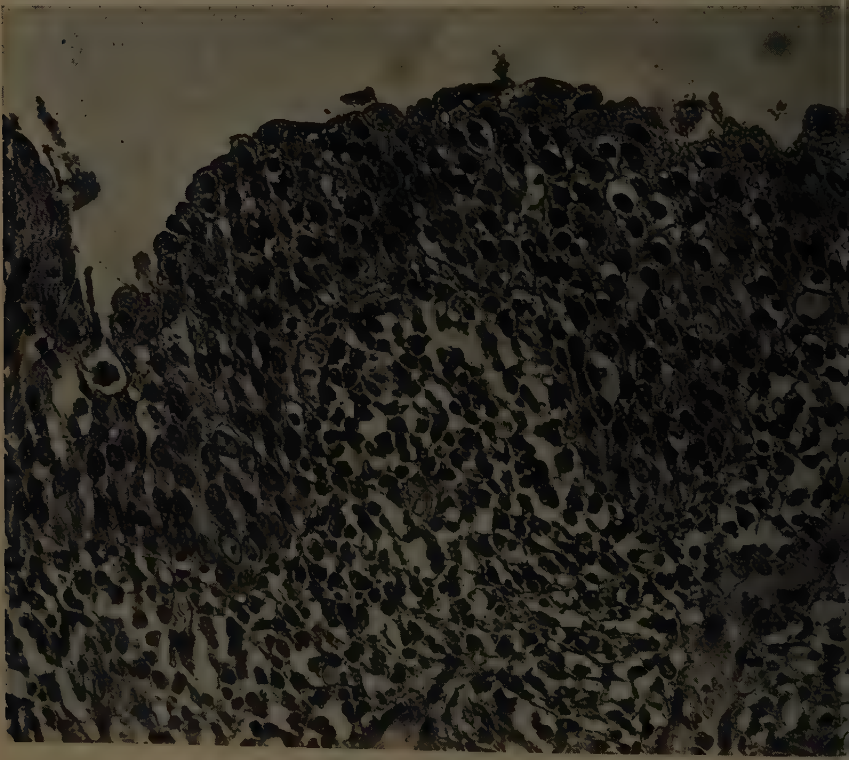


FIGURE 33. Histologic section. Human cervix carcinoma *in situ*. Hematoxylin and eosin. ×300

Atypia. In a lesion showing marked atypia associated with cancer *in situ* (FIGURE 28), there appear to be about the normal concentration of cytoplasmic mass, but the nuclei had relatively little mass except for the concentrated mass of mitotic figures or bits of nuclear chromatin. The hemotoxylin and eosin section (FIGURE 27) showing the nuclear sparcity of chromatin would suggest these findings.

Carcinoma in situ. In a case of diffuse, focal carcinoma *in situ*, two cervical glands adjacent to a normal gland showed extensive involvement with the carcinoma (FIGURES 29 and 30). In both glands, the cytoplasm of the carcinoma *in situ* cells had less concentration of mass than did the normal mucous glands. In one, the nuclei had more and, in the other, nuclei had less mass concentration than did the mucous gland cells.

In another case, the carcinoma *in situ* involving the gland (FIGURES 31 and 32) had less cytoplasmic and nuclear mass concentration than did the mucous gland cells.

In another case, carcinoma *in situ* of the exocervix had less mass (FIGURES 33 and 34) than had the adjacent connective tissue (the reverse of the usual

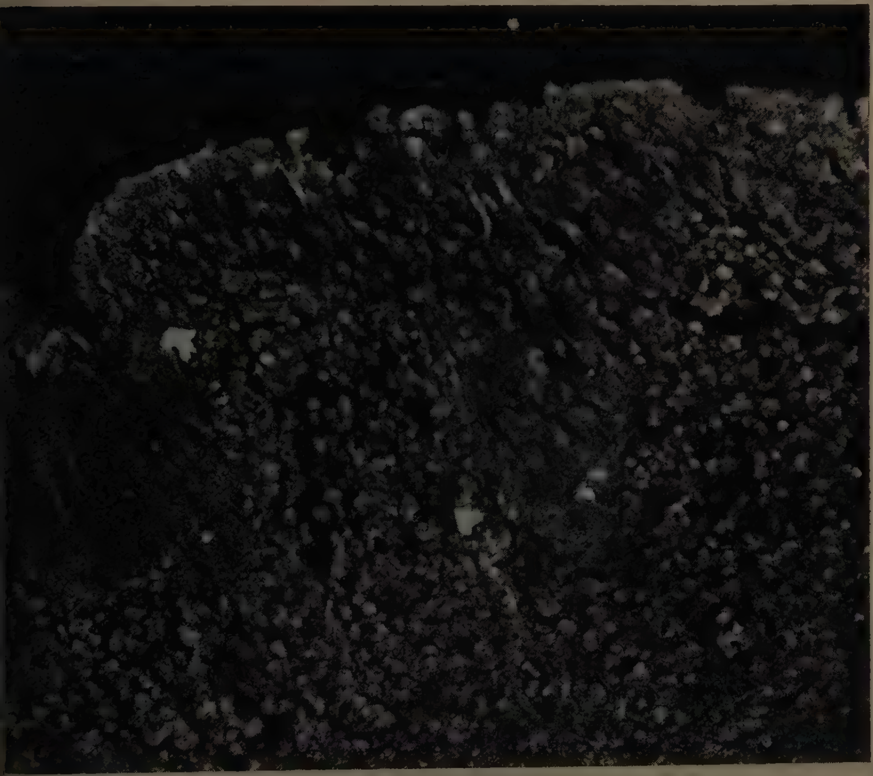


FIGURE 34. Microradiograph, section adjacent to that of FIGURE 33. Relatively little cytoplasmic or nuclear mass in the carcinoma compared to connective tissue or red blood cells. ×300.

relationship) and less than the normal cervical epithelium in the same sectior

Thus, although our material is limited, there is no substantial indication i it that there is a great increase of mass—either cytoplasmic or nuclear—in th carcinoma *in situ* cells of human uterine cervical cancer. It is suggested b FIGURES 29 and 30 that possibly, on more extended analyses, there will t found populations of carcinoma *in situ* cells, some with more and some wit less nuclear mass concentrations, and that statistical analysis of a large num ber of cells will be necessary before one could determine whether the mea masses are greater or less than in normal cells. So far, the indications ar that there are not great mass increases in cancer *in situ*, at least as reveale by this technique. The present indications are that cancer *in situ* cells of th human cervix uteri have less cytoplasmic mass than normal analogous cervic cells.

Recent technical advance. Recently Combée and Engstrom[27] have describe a new, simplified X-ray tube that is a sealed-off miniature tube with a ve thin beryllium window. Such equipment does away with the necessity vacuum pumps and reference standards and represents a great technical ac vance in the method. Such a tube is being compared to the conventional ty in this laboratory.

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QUANTITATIVE CYTOLOGY AND CYTOPATHOLOGY: NUCLEIC ACIDS AND PROTEINS IN THE MITOTIC CYCLE OF NORMAL AND NEOPLASTIC CELLS*

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It is generally thought that an initiating stimulus that produces a malignant neoplasm creates some hereditary transmissible change in the cancer cell that is passed through successive generations by cell division and contributes in some measure to the driving force for reduplication. For example, the continued presence of such agents as transient ionizing radiations or chemical carcinogens that undergo successive dilutions in each cell division is clearly not required for perpetuation of the cancers that these agents induce. Among exogenous carcinogenic factors, only those that are self-duplicating are likely to provide, by themselves, a perpetual stimulus for cell division, and this would be possible only if the reproductive capacity of the agent kept pace with that of the parasitized cell. Thus, under circumstances of cancer pathogenesis that generally pertain, it is necessary to consider the presence of an hereditary transmissible factor. This hereditary factor is currently sought in the genetic material of the nucleus and the chromosomes and in certain particulates of the cytoplasm. We shall consider, therefore, in some detail, knowledge concerning the structure and the composition of these intracellular components in normal and neoplastic cells. This will lead us directly to a discussion of the cytochemistry of those most important protoplasmic constituents, the nucleic acids and the proteins.

1. *The Normal Cell*

(*a*) *Nucleus and chromosomes.* The fundamental background underlying the chromosome theory of heredity evolved during the nineteenth century. Miescher[60] discovered in the nucleus the class of compounds that are now designated by the generic name *nucleic acids.* Flemming[24] observed that the framework of the nucleus was stained with basic dyestuffs. He christened this stainable material *chromatin*, observed the regularities of its behavior in cell division, and suggested that chromatin and, by implication in his work, chromosomes also were either identical with, or a carrier of, nucleic acids.

Evidence suggesting a relation between a specific somatic character and a particular chromosome was followed in the early part of the twentieth century by the concept, developed by Morgan[65] and his associates, that chromosomes, the physical basis of heredity, were aggregates of smaller units, the

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[*] This material was taken from the author's chapter "Cancer: Biology, Biochemistry, and Pathology," appearing in *Analytical Pathology* (McGraw-Hill Book Co., Inc., New York, N. Y., in press), and is used with the permission of the publisher.

The investigative work of the author has been supported in part by Research Grant C-1345 from the National Cancer Institute, National Institutes of Health, United States Public Health Service, Bethesda, Md., and by grants from the Damon Runyon Memorial Fund, New York, N. Y., the Elsa U. Pardee Foundation, Midland, Mich., and the Fannie E. Rippel Foundation, Newark, N. J.

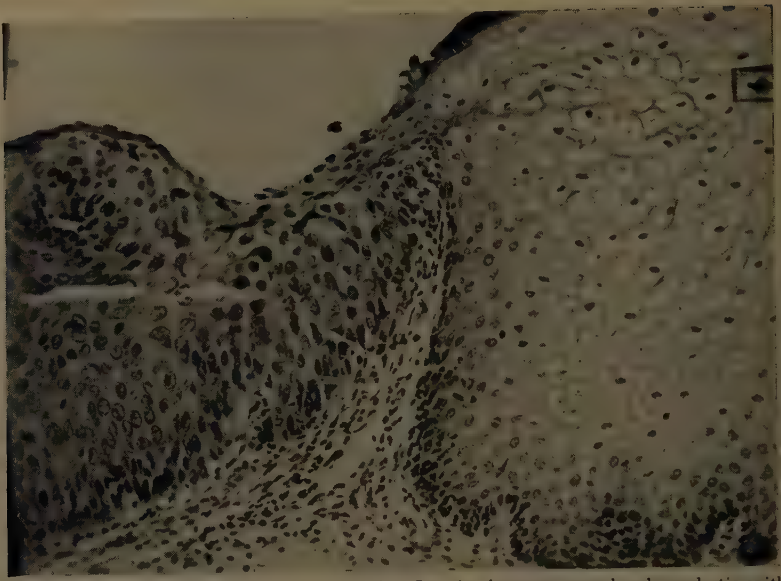

FIGURE 1. *In situ* carcinoma of the uterine cervix. Junction between normal and neoplastic epitheliu
H. and E.

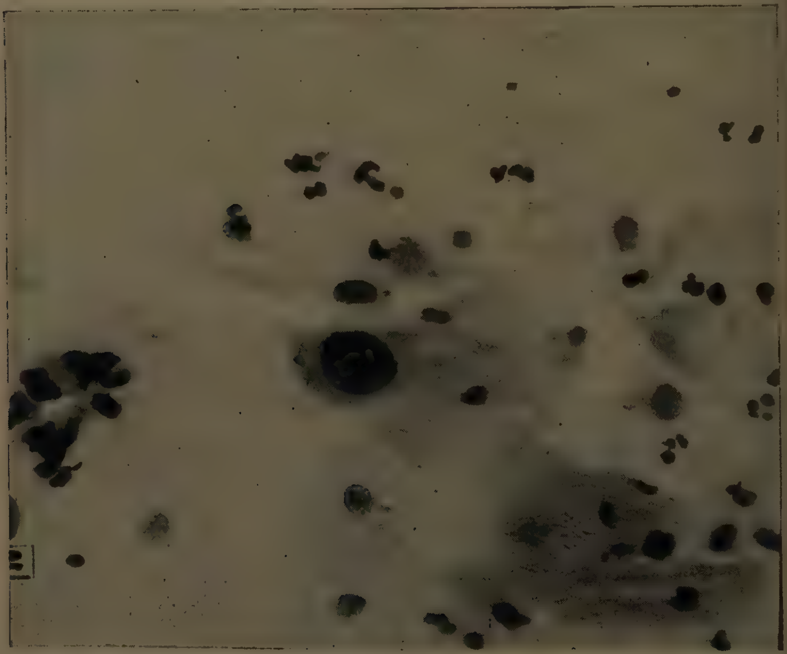

FIGURE 2. Carcinoma cell, squamous epithelial cells, and leucocytes that exfoliated from the lesion show
FIGURE 1. Papanicolaou smear.

genes, which occupied definite loci within the chromosomes. These loci achieve morphological expression in the cross-band structures of giant chromosomes, as shown by the cytogenetic studies of Painter[67] and others. Other investigations during this period were directed toward the interrelation of chromosome number, nuclear size, and cell size. Hertwig[29] called further attention to the relationship that generally exists between the size (in theory considered as the mass) of the nucleus and that of the cell, which we now know as the relative constancy of the nucleocytoplasmic ratio. Boveri[10] showed that, in lower animals, the nuclear size was proportional to the chromosome number of certain cells which, as disclosed by a study of mitotic figures, contained one, two, or four sets of chromosomes. Jacobj[37] observed that the nuclear volumes (rather inaccurately computed from two-dimensional or areal measurements) in tissues of higher animals sometimes gave frequency distribution maxima bearing a ratio 1:2:4, and so on. Despite the inherent inaccuracy of this early work, as we now recognize, cells in mammalian tissues do possess attributes that could account for a geometrical progression in nuclear volumes, namely an increase—occurring as an even integral multiple—either in the number of chromosomes (called polyploidy) or in the number of strands in each chromosome (polyteny).

The next important developments in the study of the nucleus and chromosomes proceeded in succession along chemical and physical lines. In 1924, Feulgen and Rossenbeck[23] described the application of the Schiff test for aldehydes to microscopic preparations that were subjected to acid hydrolysis. Feulgen had previously shown that thymonucleic acid, subsequently proved to contain deoxyribose and thus to be generically a deoxyribonucleic acid (DNA), reacted positively under this condition. Within the ensuing 10 years or so, about 400 papers utilizing this reaction appeared and established that the nuclear chromatin and the chromosomes of animal and plant cells contained most, if not all, of the cellular DNA.

The development in physics, or in biophysics, took place at the Karolinska Institute in Stockholm, Sweden, in the laboratory of Caspersson.[13] The specific absorption spectra of cells was measured by ultraviolet microscopy and shown to be due to nucleic acids. This microscopic technique, along with the Feulgen reaction for DNA, was used to demonstrate that, aside from DNA, a cell also contained ribonucleic acid (RNA) which, while predominantly localized in the cytoplasm, was present also in the nucleolus and was conspicuous in amount in both the cytoplasm and the nucleolus of cells engaged in intense protein-synthetic activity. Thus, the idea evolved that RNA played a key role in protein synthesis. The observation that the quantity of nucleic acid, principally DNA, in a chromosome was maximal during mitosis suggested to Caspersson that there was a connection between the duplication of genes and the presence of nucleic acids. Further support for this view was obtained from the study of giant chromosomes of *Drosophila*. The ultraviolet microabsorption analysis showed that nucleic acids were localized principally in the crossband structures and that alterations in the content and distribution of chromosomal nucleic acids could be correlated with genetic change. This

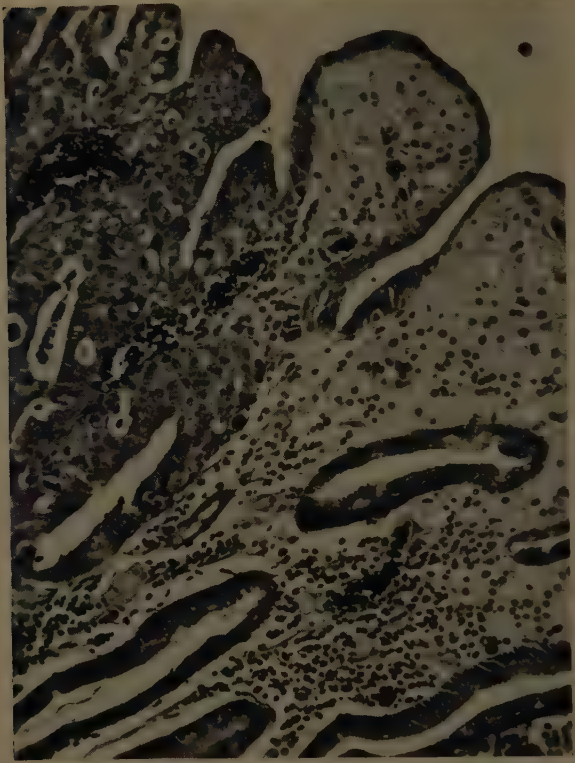

FIGURE 3. *In situ* carcinoma of the stomach. Junction between normal and neoplastic epithelium. H. a E.

observation led Caspersson and Schultz[15] to the conclusion that nucleic acid principally DNA, were prerequisites for the reproduction of genes. Asid from the study in metazoan tissues of the role of DNA in the partition c hereditary material during cell division and of the role of nucleolar and cyto plasmic RNA in the formation of cellular proteins, the Caspersson scho extended their observations to yeast cells, bacteria, and viruses, reaching i each case similar conclusions concerning the functional significance of nucle acids.

A new line of investigation pertaining to the functional role of DNA i genetic mechanisms was opened up in 1944. Avery, MacLeod, and McCarty studying the process of bacterial transformation that had been described b Griffith, showed that purified material obtained from encapsulated pneumo cocci and capable, upon contact with unencapsulated cells, of inducing e capsulation of the same serological type as that possessed by the donor cel had the chemical properties of DNA. The absorption spectra, viscosit elementary analysis, nitrogen-to-phosphorus ratios, and purine, pyrimidin and deoxyribose contents of the transforming agents, were typical of tho for DNA. As shown by Hotchkiss,[33] if any protein at all was present, t

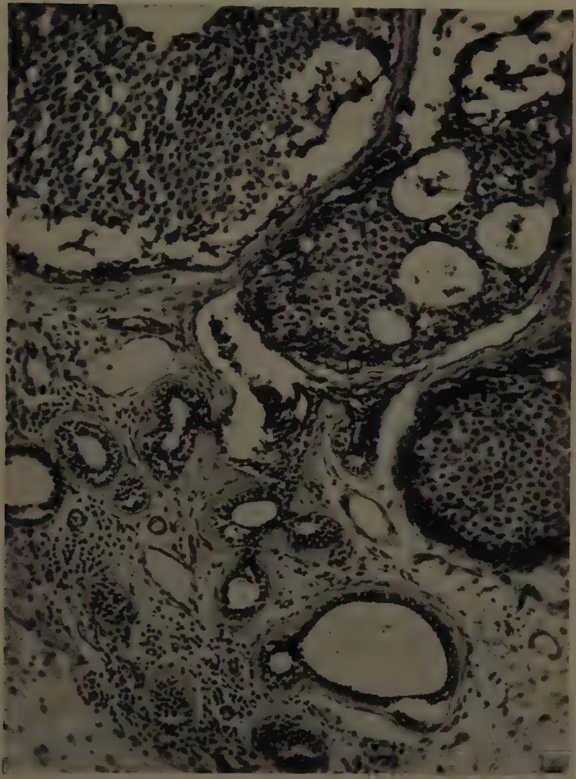

FIGURE 4. *In situ* duct carcinoma of the breast. Margin between normal and neoplastic epithelium. H. nd E.

mount could be no more than 0.02 per cent at most. About 40 examples of acterial transformations are now known, including among them the development of drug resistance to three antibacterial agents, penicillin, streptomycin, nd sulfanilamide.[33]

The implication from this work is that DNA can have a variety of different tructures with which are associated specific biological activities. Aside from timulating a great deal of chemical and biochemical work and suggesting a ole of DNA in genetic mechanisms, the study of bacterial transformation led o another discovery. Boivin,[9] cognizant of the ability of DNA to produce nheritable change in bacteria and proceeding on the theory that DNA, if a enetic constituent, should show a quantitative constancy in relation to the ucleus similar to that displayed by chromosomes, determined the average ontent of DNA per nucleus in bovine tissues by biochemical analysis. The onstancy of content anticipated for a genetic constituent was verified, and he amount of DNA in diploid somatic nuclei was found to be twice that in aploid sperm nuclei, again in accordance with expectation. These observaions have been extended by the Vendrelys[90] and by Mirsky and Ris[63] to in-

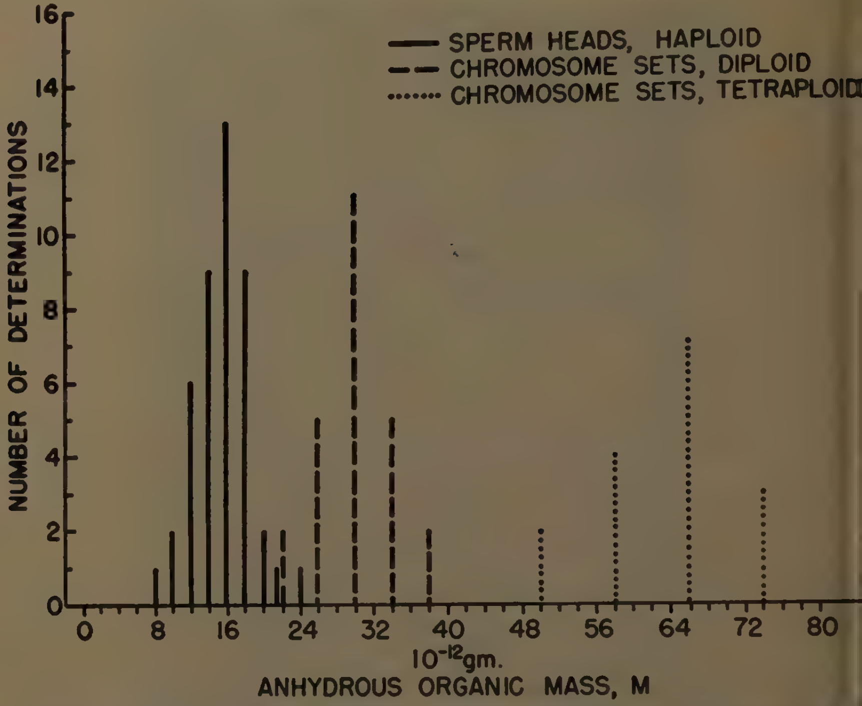

FIGURE 5. Frequency of occurrence (vertical axis) of certain values of organic mass (horizontal axis) f
mouse sperm nuclei and for prophase germinal chromosomes comprising two and four sets of chromosomes. T
modal or the average masses occur in a geometrical progression, M: $2M$:$4M$, where M, the average mass of t
sperm nucleus, is 16×10^{-12} gm. as determined by microinterferometry. From Mellors *et al.*[56]

clude somatic and germinal cells of a variety of mammals, birds, fish, an
amphibians.

If DNA is the genetic material, it is of interest to inquire into the numeric
correspondence between the DNA molecules and the genes. A cell nucle
containing Q gm. of DNA having a molecular weight W will have $Q/W \times$
molecules of DNA, where N is Avogadro's number, 6×10^{23}, and W is abou
10^6. In a normal diploid nucleus of mammalian tissues, Q is about $6 \times 10^{-}$
gm., so that the number of molecules of DNA is approximately 36×10
The estimate of the number of genes in the mammalian nucleus can be o
tained from that for *Drosophila*, about 10^3 to 10^4,[21] by multiplying by 1
there being that many more chromosomes in a mammalian cells, to obtai
the rough estimate, 10^4 to 10^5 genes. From this calculation, it appears tha
the DNA molecule:gene ratio is appreciably greater than one.[49]

The microabsorption analysis of individual nuclei stained with Feulgen
reaction for the relative content of DNA has been carried out by Ris an
Mirsky,[79] Swift,[88] Pollister,[75] and others on a variety of animal tissues. Th
frequency-distribution of DNA content has maxima occurring in a geometr
ratio, 1:2:4 *etc.*, as anticipated for nuclei containing one, two, four, or mo
sets of chromosomes. In the mouse, for example, sperm have a nuclear DN

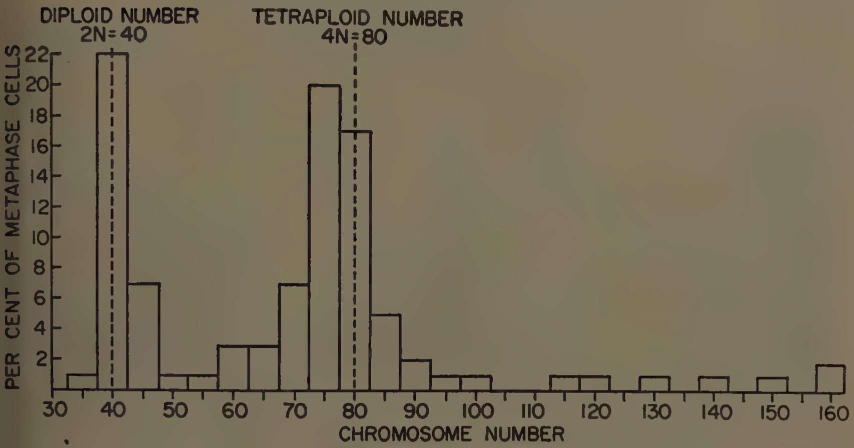

Figure 6. Frequency distribution of chromosome numbers counted in over 1,000 dividing cancer cells in various types of ascites tumors in the mouse. Lymphomas are principally near-diploid in number whereas carcinomas and sarcomas are mostly near-tetraploid. Composite data taken from Levan and Hauschka.[45]

content equivalent to one set of chromosomes; the spleen, intestinal mucosa and renal tubules, equivalent to two; the thymus gland, two and four; and the liver and the pancreas, two, four, and eight. In certain tissues in which there is appreciable mitotic activity, the nuclear DNA contents are found to lie between, as well as on, the geometric maxima, as shown by Swift and suggesting to him that DNA synthesis occurs in the resting nucleus prior to the prophase of mitosis, although there is not absolute agreement in this interpretation, as indicated by the work of Pasteels and Lison.[70]

To the interrelation of chromosome number, nuclear size, and cell size, which attracted the interests of early cytologists, is thus added the nuclear content of DNA. Any idea that the cellular nucleic acid content is an esoteric concept in routine cytology can be dispelled by the work of Michaelis,[59] who has shown that the simple basic staining methods empirically developed by cytologists provide relatively selective techniques for the demonstration of nucleic acids, provided that certain specifications, frequently met in practice, are fulfilled. Brachet,[11] for example, using the methyl green-pyronin stain described many years ago by Unna and Pappenheim, together with enzymes (nucleases) that digested RNA and DNA, independently reached many conclusions concerning the functional role of nucleic acids similar to those obtained by the Caspersson school with technically more intricate methods. Moreover, a substantial amount of applied morphologic cytology, perhaps more than is generally realized, utilizes in one way or another visual estimations of the cellular and, particularly, the nuclear content and distribution of nucleic acids, a subject to which we shall return in the discussion of the cancer cell.

Were we to conclude the discussion on the structure and the composition of the nucleus and chromosomes without a consideration of the protein fraction, we should be neglecting as much as 75 per cent of their quantitative organic composition.[56] This fact alone would seem to justify a constant and

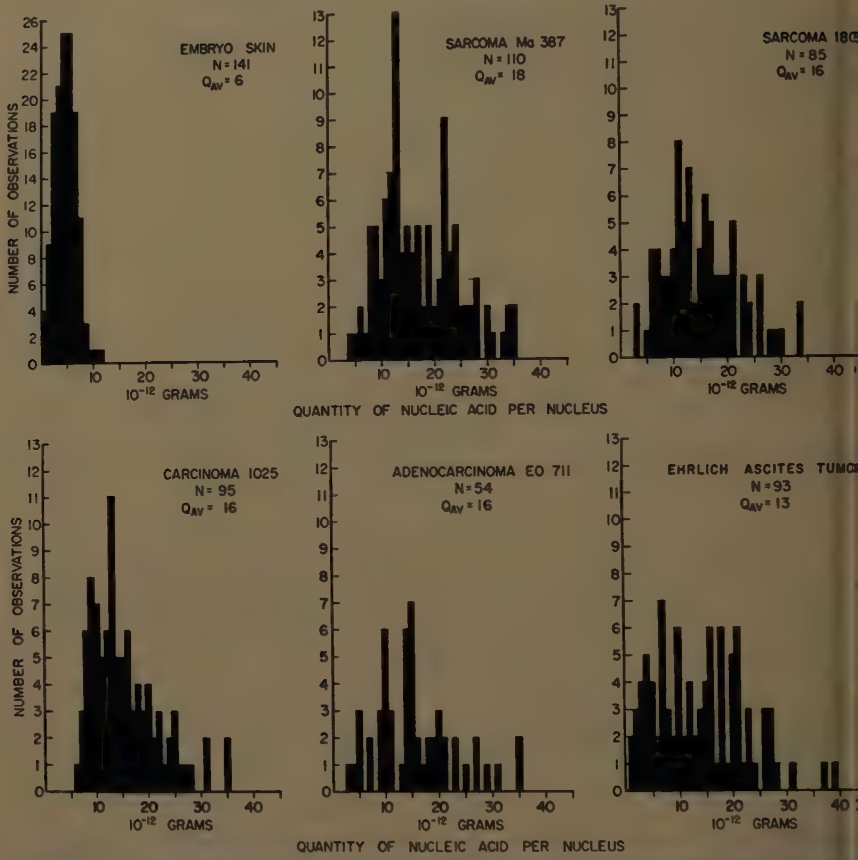

FIGURE 7. Frequency distribution of certain quantities of nucleic acid per nucleus in skin (spleen yie similar data) and in transplantable sarcomas and carcinomas of the mouse. The average value, Q_{AV}, as me ured by intraviolet microabsorption is 6 × 10^{-12} gm. for presumably diploid somatic nuclei (skin, spleen), wh the high values in the tumor cell nuclei are probably associated with polyploidy. From Mellors *et al.*[52]

searching interest in nuclear proteins but, paradoxically, the expenditure effort and the knowledge gained on this subject have been small. As Mazia has stated in his comprehensive review on the physiology of the nucleus, fro a biological viewpoint every organic constituent of the nucleus deserves e amination as the potential carrier of genetic specificity. Historically, t earliest work on nuclear proteins was done by Miescher,[60] whose organ analysis of fish sperm nuclei was partitioned between nucleic acids, a bas protein called protamine, a residual protein material, and lipids. Miesch thought that the nucleic acid was too simple in composition to be the gene component. Today, of course, this objection does not apply in view of t recognition that qualitative, quantitative, and sequential differences in t purine and pyrimidine bases of nucleic acids can exist and, moreover, th the spatial geometry of a giant molecule, having, as does DNA, a molecul weight of one-half million to three million, can be of decisive functional s

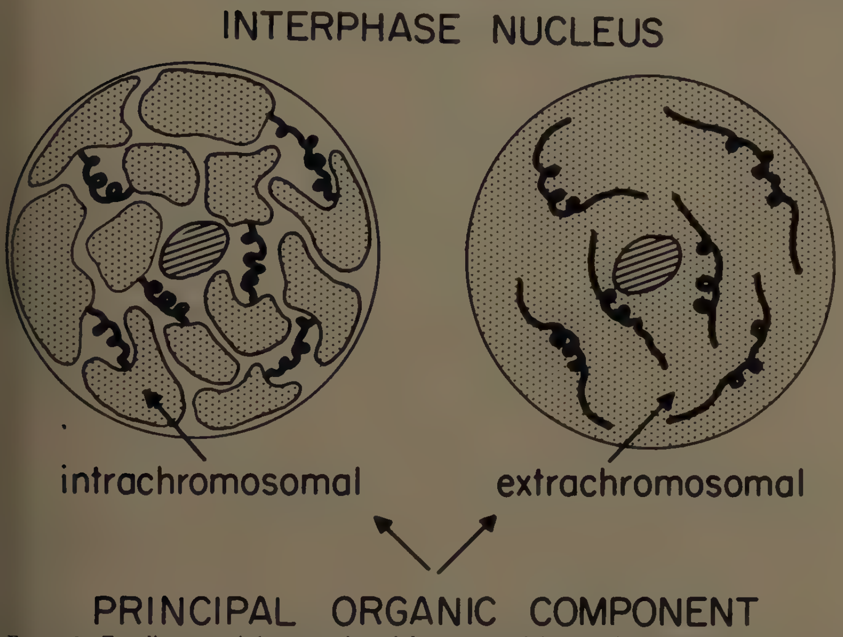

FIGURE 8. Two diagrammatic interpretations of the structure of the interphase nucleus. In the one, the principal organic mass is intrachromosomal; in the other, extrachromosomal. From Pollister.[73]

nificance, as shown by the specificity of immune globulins. It is equally mportant to recognize, however, what we do not know about DNA. Hotchkiss,[33] in his recent Harvey lecture, states this most succinctly: "It is safe to say that not only do we know nothing about the chemical principles upon which specificity of DNA is operative, but furthermore, if one takes it to be a genetic determinant, there is still very little idea what features of enzyme proteins or other compoents are actually genetically determined. . . ."

Miescher also rejected the basic protein-fraction as the genetic component on the basis of simplicity of structure, a view that is fully concordant with existent knowledge. It has been known since Kossel's[40] time that the nuclear basic protein varies greatly in composition, particularly in arginine content, in different tissues of the same animal. In fish sperm, during maturation, the basic protein actually changes from a histone to a protamine. Thus, it is unlikely that the basic protein is the genetic component in itself. Analytical data show that the nuclear content of basic protein is about equivalent to hat of DNA, and they suggest that the DNA exists as a DNA-histone.[61, 62]

After removal of DNA-histone from nuclear proteins, a nonhistone protein emains. This fraction, characterized by insolubility and a high content of ryptophan and sulfur-containing amino acids, is called chromosomin by the Stedmans[84] and residual protein by Mirsky and Ris.[62] Irrespective of name, his fraction is regarded by both groups of investigators as one of the most mportant chemical constituents of the chromosome: by the Stedmans as the hromosome itself in the resting nucleus, floating as a threadlike structure in

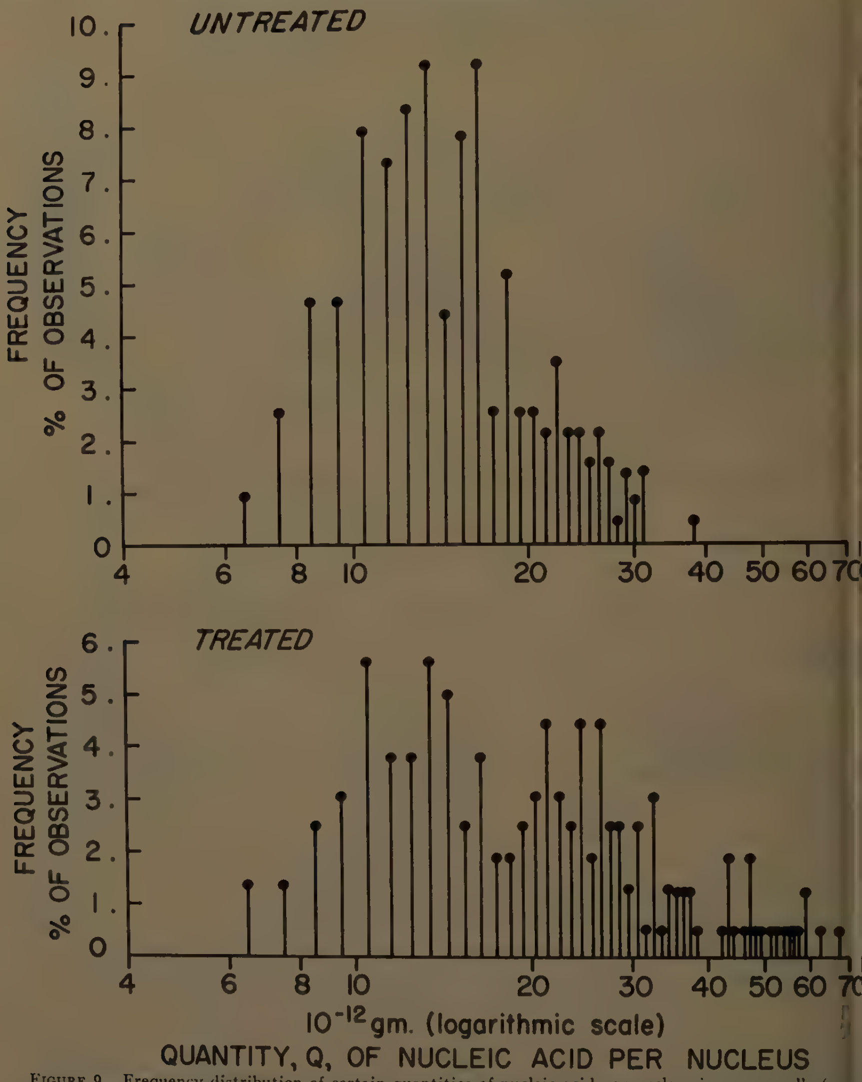

FIGURE 9. Frequency distribution of certain quantities of nucleic acid per nucleus in cancer cells (mou carcinoma 1025) in untreated animals and in those treated with triethylene melamine (TEM), lower graph, in dosage which grossly arrests tumor growth. The occurrence in the treated cancer cells of higher values of nucle acid per nucleus (for example, values greater than 16×10^{-12} gm.) is statistically significant (chi square = 8) relation to the untreated cancer cells. From Mellors and Sugiura.[57]

a nuclear sap containing DNA-histone; and, by Mirsky and Ris, as the bas of the linear order of the genes in the chromosome. Pollister[73] considers bot conclusions to be somewhat speculative in the present state of knowledge, an he has not personally succeeded in differentially removing DNA-histone, fc example with 1 M NaCl as used by Mirsky and Ris, without also causin dissolution of isolated chromatin threads. Moreover, Mazia states tha when the residual protein of a chromosome is digested by pepsin, which leaves behind the DNA and at least some of the basic protein, a continuous structure having the structural characteristics of the chromosome remains. In contrast with the relative constancy of nuclear DNA-histone, residual protein appears to be a variable component and also to be intimately associated with RNA, according to the data of Mirsky and Ris, suggesting a dual chemical organization of the chromosomes.

Caspersson was impressed with the nuclear and the chromosomal content of proteins, stating as he did that the process of mitosis was dominated by a redistribution of the proteins in the nucleus, but adding also that, even with the highest precision in the ultraviolet microabsorption measurement, it was not possible to make an exact determination of the amount or the character of the proteins in the mitotic cycle. Pollister and Leuchtenberger,[74] using the cytochemical version of the Millon reaction for tyrosine- and tryptophan-containing protein, were led to the conclusion that, aside from residual protein, resting nuclei contain a relatively larger amount of another type of non-histone protein, which apparently is lost in some of the nuclear-isolation procedures used by biochemists. Schrader and Leuchtenberger,[81] using the Millon and the Feulgen reactions, found the nuclear volumes in cells of an nsect to be proportional to the content of tyrosine-containing protein, but not of nucleic acid, in the nucleus. Thus some form of nuclear protein appears to be a determinant of nuclear size in this cytological material, a concept that is applicable, in some extent at least, to mammalian cells.

A recent development in microscopy—the cytological application of the nterference microscope—[5, 6, 20, 54, 56] now enables one to determine for the irst time the weight of unstained, and even living, cells and cellular components by precise optical means, and it provides also a direct image of structural details with excellent and controllable contrast and high resolution (FIGURE 10). While the application of the method to the analysis of chromoomes has just begun, and although the data have been principally derived rom one species of animal, the mouse, some concepts of cytology not heretofore proved have already been confirmed (FIGURE 5) and additional new deas appear to be forthcoming. Stated in simplest terms, the dry weights f sperm nuclei, presumably containing one set of chromosomes, and of mitotic hromosomes composing two and four sets have been measured and found to ear the geometrical ratio $M{:}2M{:}4M$, where M, the organic mass of one set f 20 germinal chromosomes, is 16×10^{-12} gm. This is in accordance with xpectations, provided *all* of the organic material of the chromosome is hereditary material. Calculations show, moreover, that DNA could constitute nly about 20 to 25 per cent of the organic material. Moreover, the constancy n the content of total organic material in each chromosome set, in a given ne of germinal cells, and the equipartition of this material between chromoomes of dividing cells is found to be regulated just as precisely as is the content of DNA. While the sequential chemical fractionation of the chromoome *in situ* is feasible by this analytical method, the work has not yet been arried out, but the fact that the DNA:histone ratio is generally accepted to

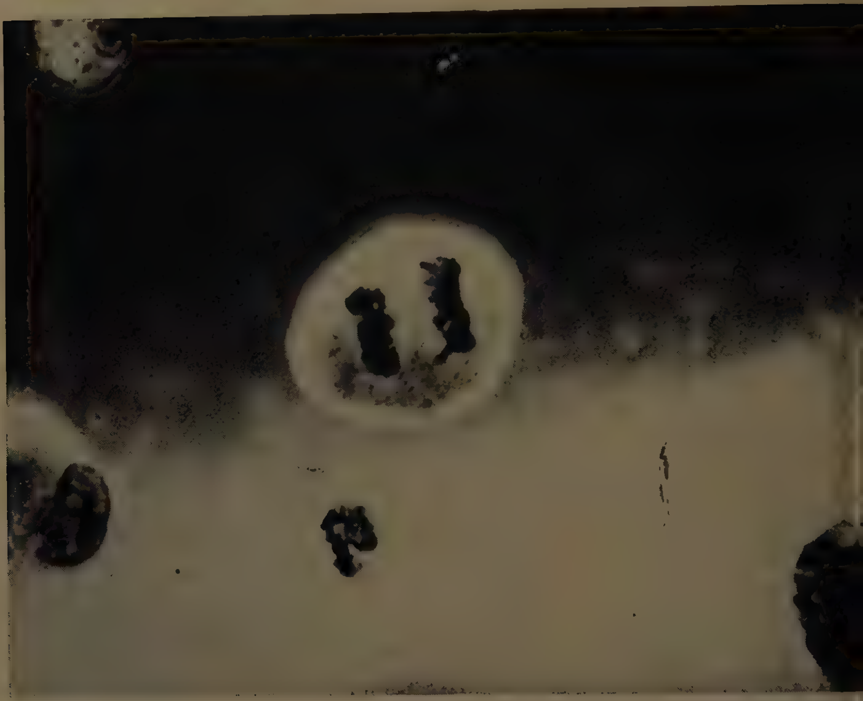

FIGURE 10. Anaphase chromosomes in a dividing cancer cell (mouse sarcoma 180, unfixed and unstaine photographed with the multiple-beam interference microscope. From an analysis of the photographic density the interference pattern the dry weight of the two groups of chromosomes, each destined for a daughter cell, c be determined.

be near unity yields this probable partition of organic mass in fat-free germin chromosomes of the mouse: DNA, 25 per cent; histone, 25 per cent; and no histone protein associated with RNA, 50 per cent. Evidence previously cite has suggested that the nonhistone protein represents the quantitative variable component of the nucleus in different tissues of the same anima If this is confirmed by the interferometric analysis of chromosomes *in sit* then a dual organization of chromosomes will be further established. Bo components undergo equipartition in cell division. One, the DNA-histon is relatively constant per chromosome set in all cells of a particular anima and the other, RNA-residual protein, is variable. If this is so, does th variable component play a decisive role, as has been suggested, in the fun tional differentiation of cytoplasm that distinguishes one type of cell fro another and, since lack of differentiation is a common attribute of a canc cell, does the variable component have bearing on the genesis of cance Clearly, this line of thought leads us to a fundamental research frontier, whe decisive answers must be eagerly but patiently awaited.

(*b*) *Cytoplasm.* In relation to the extensive literature dealing with th recent expansion of knowledge concerning the interrelation of the biochemist and the biomorphology of cytoplasmic particulates, only the briefest comments can be made here. By the use of methods of cell fractionation, introduced by Bensley and Hoerr[7] and by Claude,[17] a homogenate of an organ, such as the liver, can be partitioned into nuclear and cytoplasmic fractions constituting about 10 and 90 per cent, respectively, of the total cellular protein. The cytoplasmic fraction in turn can be separated into components that constitute, in terms of total cellular protein, approximately the following proportions:[78] mitochondria, or large granules, 30 per cent; microsomes, or small granules, 20 per cent; and supernatant fluid, 40 per cent. It has been shown by biochemists, among them Schneider,[80] Hogeboom,[32] and Lehninger,[42] that mitochondria contain a large proportion of the total tissue content of oxidative, hydrolytic, and synthetic enzymes, including, as a key system, the enzymes of the Krebs citric acid cycle, perhaps the most efficient system yet discovered in the cell for the conversion of oxidative energy into readily utilizable phosphate bond energy. A corollary of this, as shown by the studies of Lazarow and Cooperstein,[41] is the explanation in terms of enzymatic activity of the reason for the intravital staining of mitochondria with Janus green B, a staining technique familiar to cytologists since its discovery by Michaelis in 1900. Finally, a certain pattern of organization, resolved now by the electron microscope nearly to the macromolecular level, has been found by Palade[69] and by Sjöstrand and Rhodin[83] to provide a structural arrangement that is both consistent with, and apparently a prerequisite for, a spatial distribution that permits enzyme units to carry out so many diverse functions in such a small space.

2. *The Cancer Cell*

(*a*) *Structure and composition.* If we were to ask a pathologist what are the identifying microscopic characteristics of a particular type of cancer, he would say, in effect, a familiar histological and cytological appearance that has a time-proven correlation with a certain pattern of anatomical growth and clinical behavior. Fully appreciative of the practical importance of viewing the whole pathological picture, it is our intention, nevertheless, in this short section to look at the cancer cell itself.

The size of the cancer cell and, particularly, the size of its nucleus and nucleolus, both of which are frequently found to be enlarged relative to dimensions in the homologous normal cell, has attracted the interest of microscopists for more than a century, as Hoffman's[31] historical survey has shown. We are ready, from the observations in general cytology that have been sited, to accept, and to expect, an increase in the size of the nucleus of a cell if, for example, the chromosome number is found to be increased. While various alterations in the chromosomes of cancer cells have been discussed repeatedly during the last half-century from the point of view of both cause and effect, the conclusions justified by quantitative data obtained on a statistically adequate and cytologically favorable sample are as follows. In cancer cells, the more frequent values of chromosome number fall within a relatively narrow range, with the numerical variation (aneuploidy) more or less comparable to

TABLE 1

CHROMOSOME NUMBER OF CANCER CELLS IN VARIOUS TYPES OF NEOPLASMS OF MAN AND ANIMALS AND IN NEOPLASTIC CELLS OF PLANTS

Neoplasms	Material	Chromosome number		Reference
		Near diploid*	Near tetra- or poly-ploid	
In man				
Epidermoid carcinoma, lip	Surgical		+	(a)
Epidermoid carcinoma, cheek	Biopsy		+	(b)
Epidermoid carcinoma, cervix	Tissue culture		+	(c)
Liposarcoma, arm	Tissue culture		+	(d)
Malignant melanoma	Tissue culture		+	(d)
In the mouse				
Carcinomas, six types	Ascities and solid		+	(a) (e) (f
Carcinomas, one type	Ascites	+		(f)
Sarcomas, six types	Ascites and solid		+	(e) (f)
Lymphomas, three types	Ascites	+		(e) (f)
Lymphomas, one type	Ascites		+	(e) (f)
Other				
Rat sarcoma, two types	Solid and tissue culture		+	(g) (h)
Rat sarcoma, one type	Ascites	+		(i)
Chicken sarcoma			+	(a)
Plant crown gall disease			+	(j)

* Diploid number (2N) for man is 48, for mouse 40.

† Reference: (a) Levine;[46] (b) Heiberg and Kemp;[28] (c) Hsu;[35] (d) Hsu;[34] (e) Hausch and Levan;[27] (f) Levan and Hauschka;[45] (g) Lewis and Lockwood;[47] (h) Hirschfeld and Kl Rawidowicz;[30] (i) Yoshida;[92] and (j) Winge.[91]

that now recognized as existing in many normal cells. Moreover, while so types of cancer cells have a most frequent (modal) chromosome number th is near-diploid, namely, 2N plus or minus a few chromosomes, similar normal diploid somatic cells, many types of cancer cells have a near-tet ploid number that is near 4N, or twice the diploid chromosome number. T is shown in TABLE 1, where it is seen that chromosome numbers that are eit near-tetraploid or polyploid, that is, substantially greater than diploid, found in tissue sections of epidermoid carcinoma of the lip and of the buc mucosa in man and in tissue cutltures of human cervical epidermoid c cinoma and liposarcoma. While the modal value of malignant melanoma tissue culture is diploid, polyploid values exist, and several other types human cancer cells *in vitro* are also polyploid according to the studies of Hs Tissue cultures and ascites tumors provide the most favorable material chromosome counts because individual, well-spread cells can be studi Data obtained on mouse ascites tumors, particularly by Hauschka and

TABLE 2

INCREASED NUCLEAR CONTENT OF DNA, OR WHERE INDICATED NUCLEAR TOTAL NA, IN CANCER CELLS OF VARIOUS TYPES IN MAN

Cancer	Frequency, or % of cases with increase	Reference*
pidermoid carcinoma	18/21 = 86%	
skin	10/12	(a)
upper resipiratory tract	4/5	(a)
bladder	2/2	(a) (b)
cervix, in situ (total NA)	2/2	(c)
denocarcinoma	17/28 = 61%	
colon-rectum	4/5	(a) (b)
stomach	2/4	(a) (b)
lung	2/3	(a) (b)
pancreas	1/2	(b)
kidney	2/2	(a) (b)
breast	4/10	(b)
liver	1/1	(a)
prostate	1/1	(a)
iscellaneous	2/3 = 66%	
leiomyosarcoma	1/1	(a)
leukemia	0/1	(d)
seminoma	1/1	(b)

* Reference: (a) Stowell;[86] (b) Leuchtenberger *et al.*;[44] (c) Mellors *et al.*;[53] and (d) Metais nd Mandel.[58]

evan, are given in TABLE 1 and FIGURE 6. All but one of seven carcinomas udied and all six sarcomas are near-tetraploid. Contrarily, all but one of ur lymphomas are near-diploid. Two of three sarcomas studied in the rat, chicken sarcoma, and neoplasticlike tissue in crown gall disease of plants re either near-tetraploid or polyploid.

Caspersson and Santesson[14] were the first to interpret the well-recognized lterations in the staining properties of the nucleus, the nucleolus, and cyto- lasm of cancer cells in terms of content, type, and distribution of nucleic cids, a viewpoint that has served as a stimulus for much cytochemical work n this field. Quantitative data, obtained principally by Stowell and by euchtenberger and her associates, on the nuclear content of DNA in cancer ells of various types in man are summarized in TABLE 2. In epidermoid rcinoma of cutaneous, respiratory, vesical, or uterine origin, the DNA or ne total NA content per nucleus in cancer cells is increased, relative to that n homologous normal cells in 86 per cent of the lesions analyzed. Adeno- rcinomas of alimentary, thoracic, mammary, and prostatic origin have an creased DNA content in 61 per cent of lesions studied, and miscellaneous ancers in 66 per cent. In the mouse, as shown in TABLE 3 and FIGURE 7, all f seven carcinomas, including in this a melanoma and all of four sarcomas, ave an increased nuclear content of DNA or total NA, together with in- reased nuclear RNA in all cases examined. In only one of three lymphomas, owever, is the nuclear DNA content increased, while the RNA content, hen measured, is consistently found to be elevated. These results, obtained n morphologically resting cancer cells in which chromosomes are not visible, re in general accord with those expected from the studies of chromosome

TABLE 3

INCREASED NUCLEAR CONTENT OF DNA, OR WHERE INDICATED UNDER COMMENTS, NUCLEAR RNA OR NUCLEAR TOTAL NA, IN CANCER CELLS OF VARIOUS TYPES IN THE MOUSE

Cancer	Comment	Reference*
Carcinoma		
Ehrlich	RNA also	(e)
-1509a, mammary	RNA also	(f)
Krebs—2	RNA also	(f)
-1025	total Na	(g)
-E0771	total NA	(g)
sponanteous, mammary	—	(h)
S-91, melanoma	—	(h)
Sarcoma		
-37	RNA also	(f)
MC1M rhabdomyosarcoma	RNA also	(f)
-180	—	(i)
-Ma387	total NA	(g)
Lymphoma—leukemia		
lymphoid leukemia	RNA only	(j)
DBA lymphoma	RNA only	(f)
lymphosarcoma L-1	—	(i)

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* Reference: (e) Goldberg *et al.*;[25] (f) Klein;[38] (g) Mellors *et al.*;[52] (h) Bader;[4] (i) Carr *et al.*;[12] and (j) Petermann and Schneider.[72]

number in the same type of cells in mitosis. For if, as in the normal ce the DNA content per chromosome set is relatively constant, then cancer ce having more sets of chromosomes will have more nuclear DNA. While o knowledge of the state of the resting (interphase) nucleus and of the chrom somes within it yields two alternative interpretations[73] (FIGURE 8), most c tologists believe that the nuclear organic mass is largely intrachromosoma and that the chromosomes lose their visibility in the resting nucleus, exce for certain chromatic regions, by uncoiling and swelling so as to occupy near the entire nuclear volume. Thus, a common structural attribute of a canc cell, namely an increased content of nuclear chromatin, is most logical interpreted in terms of an increased number, frequently occurring as multip sets, of chromosomes.

Although polyploidy and an increased nuclear content of chromatin a clearly not requisites for the continued propagation of a cancer, being abse from some, nuclear enlargement accompanied by a normal or elevated optic density in chromatin—in either case yielding, as a product of nuclear ar and optical density, an increased content of chromatin—is a very comm finding in early carcinogenesis. This is shown by the work of Cowdry[18] a his associates on epidermal carcinogenesis in the mouse, wherein enlargeme of nuclei and of chromosomes was observed after a relatively short period treatment with methylcholanthrene and at a substantial time before the d velopment of manifest squamous-cell (epidermoid) carcinoma.

A study of a wide variety of cancers in man, likewise, indicates that, in t earliest detectable stages of cancer, namely, that of *in situ* or preinvasi carcinoma, the feature of increased nuclear content of chromatin is frequentl although not invariably, present. This feature occurs more commonly in epidermoid carcinoma *in situ* of skin and uterine cervix; less conspicuously in adenocarcinoma *in situ* of stomach, gall bladder, and breast, seminoma *in situ* of the testis, and islet-cell carcinoma *in situ* of the pancreas; and regularly in the morphology of malignant cells that exfoliate from *in situ* carcinoma of the cervix (FIGURES 1 to 4). Moreover, if the stage of tumorigenesis is examined at a step antecedant to cancer, in the skin and the mucous membranes in precancerous keratosis and leukoplakia, a conspicuous morphological feature is also found, namely so-called malignant dyskeratosis or its equivalent,[1] in which nuclear alterations such as those under discussion are present. Since the precancerous lesion is a statistical, as well as a morphological, concept, signifying generally a high but no invariable association with the eventual development of cancer, it is clear that factors in addition to those attaining morphological expression are involved in the final genesis of cancer for, obviously, all precancerous lesions do not evolve into cancer. Moreover, many cancers, lacking at the time of histological recognition any interpretable relation to an antecedent lesion, appear to arise *de novo*.

The recognition that a variety of injurious physical and chemical agents can also produce nuclear alterations in normal cells similar to those under discussion appears at first glance to detract from any significance that might be attributed to these morphological changes in the genesis of cancer. However this may be, the alterations produced by these agents do merit consideration. The nuclear enlargement and the increased chromatin content that accompanies cellular injury is logically interpreted as due to polyploidy, as though reduplications or enlargements of chromosomes had been made in preparation for nuclear and cellular divisions but not successfully used. Not only extrinsic chemical, physical, and biological agents but also intrinsic factors such as hormones can increase the size of cell nuclei. For example, Arias-Stella[2] has shown that nuclear enlargement, approaching gigantism and cytologically simulating that in cancer cells, occurs in glandular epithelium of the human endometrium when viable and presumably functioning, chorionic tissue is retained therein. Furthermore, in certain benign lesions of the endocrine glands—parathyroid adenomas provide infrequent, yet notable, examples—nuclear enlargement of an extreme degree may occur in epithelial cells.

With respect to the mechanisms of polyploidization, it is now recognized that chromosome reduplication can take place within the intact, so-called resting, nucleus or during any phase of the mitotic cycle from prophase to telophase;[36] and, as shown by Biesele and his associates[8] and by Levan and Hauschka,[45] this activity is conspicuous in certain types of tumors in the mouse and achieves expression later in mitotic figures having supernumerary or multistranded chromosomes. An alternative explanation for the origin of a polyploid nucleus is that the spindle mechanism fails to facilitate the orderly separation of the reduplicated chromosomes at metaphase, and the nucleus is then reconstructed with an increased content of chromatin. The actions of physical or chemical agents, among them ionizing radiations and the nitrogen mustards, which restrain the growth of certain cancers, are of interest in this regard. As a therapeutic consequence, there are at least three early mor-

phological expressions of nuclear injury, aside from a diminution in mitot
activity; namely, nuclear pyknosis, nuclear enlargement as though due
excessive hydration, and nuclear enlargement accompanied by substanti
chromasia. The following observation is illustrative of the latter change.
quantitative cytochemical study[57] of the transplantable mouse tumor, ca
cinoma 1025, disclosed that the untreated cells had polyploid, or near-tetr
ploid, nuclear contents of NA. When, however, the growth of the tumor w
grossly inhibited by the agent triethylenemelamine, which is chemically a
pharmacologically allied to the mustards, many of the cancer cells were foun
to contain more than the anticipated quantity of nuclear NA (FIGURE
The preparatory synthesis of materials, here shown by enzymatic cytochemic
studies to be nucleic acids of DNA and RNA type, had been carried out
the cell, although the materials were not utilized in mitotic division, t
mitotic index itself being much decreased. A similar cytological pictu
possibly admitting of a comparable interpretation, occurs in the early stag
of growth inhibition of other experimental tumors treated with various chem
cal agents, as, for example, in responsive sarcomas and carcinomas of t
mouse and the rat treated with 6-mercaptopurine, Aminopterin, A-methopteri
and triethylenemelamine.[16, 85, 87] These findings suggest that a discordan
in the synthesis and the utilization of chromosomal nucleic acids attains mo
phological expression in certain types of cells injured by various agen
Interestingly enough, some of these agents, such as the mustards and ionizi
radiations, have a dual action, being cancerolytic under some conditions a
cancerogenic in other circumstances. It is not to be inferred from this, ho
ever, that the primary effect of these agents is on the nucleus alone for, clear
cytoplasmic and nuclear functions are intimately interrelated.

In the cytological literature on cancer, much more attention has been giv
to the chromosomal than to the nuclear structural alterations. Clearly, t
antecedent nuclear and cytoplasmic events that influence chromosomes shou
leave a mark upon them, and the list of the morphological changes in chrom
somes of cancer cells and of the variations in the details of mitosis, in additi
to the commonly observed accelerated mitotic rate, is impressive indee
Koller[39] classifies into three types the mitotic abnormalities that occur *nat*
rally in tumors. These include changes in the structure and in the number
chromosomes and abnormalities in the mitotic spindle. Structural alteratio
in the chromosomes most commonly arise when, as a consequence of the
"stickiness," an interchromosomal bridge develops and delays, or otherwi
alters, the chromosomal separation at anaphase. Numerical changes in t
chromosome complement, as we have seen, approach balanced ploidy in t
majority of proliferating cancer cells, thus establishing a "stem-line"[48] of ce
but, in some cancer cells, the chromosome number may be highly irregul
to a degree probably incompatible with further propagation. Abnormalit
in the spindle apparatus include its partial or complete suppression and t
development of multipolar, particularly tripolar, division. Since many or
of these effects can be brought about experimentally in normal cells by vario
agents or causes, including among them X rays, low temperature, and c

hicine, these chromosomal changes are not unique for the cancer cell. The tructural attributes of chromosomes in cancer cells observed by Makino and Kanô,[48] however, may have a more far-reaching significance. While some of the chromosomes in cancer cells were found to be rod-shaped, as were those n normal somatic and germinal cells of the rat, certain chromosomes had haracteristic and tumor-specific V- and J-shapes that were never found in the host cells.

Recently, attention has been directed to two additional mitotic abnormalities of the cancer cell. Timonen and Therman,[89] in a study of normal and neoplastic tissues of the genital tract of women, found that, in relation to other phases of the mitotic cycle, there were relatively fewer prophases in carcinoma cells, which suggest that, if frequency is proportional to duration, the prophase duration is shorter in cancer cells than in normal cells. Another mitotic abnormality, said to occur particularly in cervical *in situ* epidermoid carcinoma[22] exists, in which the metaphase mitotic figure is composed of three parts: a main mass of chromosomes occurring in the equatorial plane, and two esser masses lagging behind near each polar region.

The optical measurements of the dry weight of sets of chromosomes in normal cells has already been discussed and illustrated (FIGURES 5 and 10). The analytical method has been used to weigh the nucleoprotein material, ound generally to be about 80 to 85 per cent of the total dry weight, in chromosomes of mitotic cancer cells in various transplantable mouse tumors (FIGURE 11). These mitotic cancer cells have, as their lowest value, approximately he same chromosomal mass as found in a normal (diploid) cell in mitosis and, as their most frequent value, about four times this mass, a finding that is attributed to an increase in either the number of the size of the chromosomes, or both, as future work will decide.

The dry weight of nuclear material in normal and malignant epithelial cells derived from squamous mucosas in man has been measured in preliminary work with the Baker interference microscope. The nuclear mass in the quamous cancer cell was found to be two to four times that of its normal prototype, again apparently reflecting the presence of an excess number of ets of chromosomes. The distribution of dry mass in histological sections of ormal and neoplastic tissues has been ascertained by interferometry[19] and by X-ray absorption analysis.[64]

Recently, having shown that the quantity of nuclear chromatin in squamous cancer cells exfoliating from the uterine cervix was significantly different rom that of homologous normal epithelium,[53] the cytological, cytochemical, nd opticoelectronic principles underlying the development of an automatic canning microscope for preliminary cytodiagnosis were presented.[51, 55] In ssence, the nuclear content of chromatin was measured by integrating over he nuclear area the quantity per unit area of nucleic acid, measured by atural ultraviolet absorption, or of chromatin, estimated by its combination ith a basic dyestuff that may serve as a light absorber or, if a fluorochrome, s a light emitter. This morphological attribute, when surveyed in each cell, as transformed into a simultaneous electronic event by a photoelectric canning device built into the microscope, as shown in FIGURE 12. Recog-

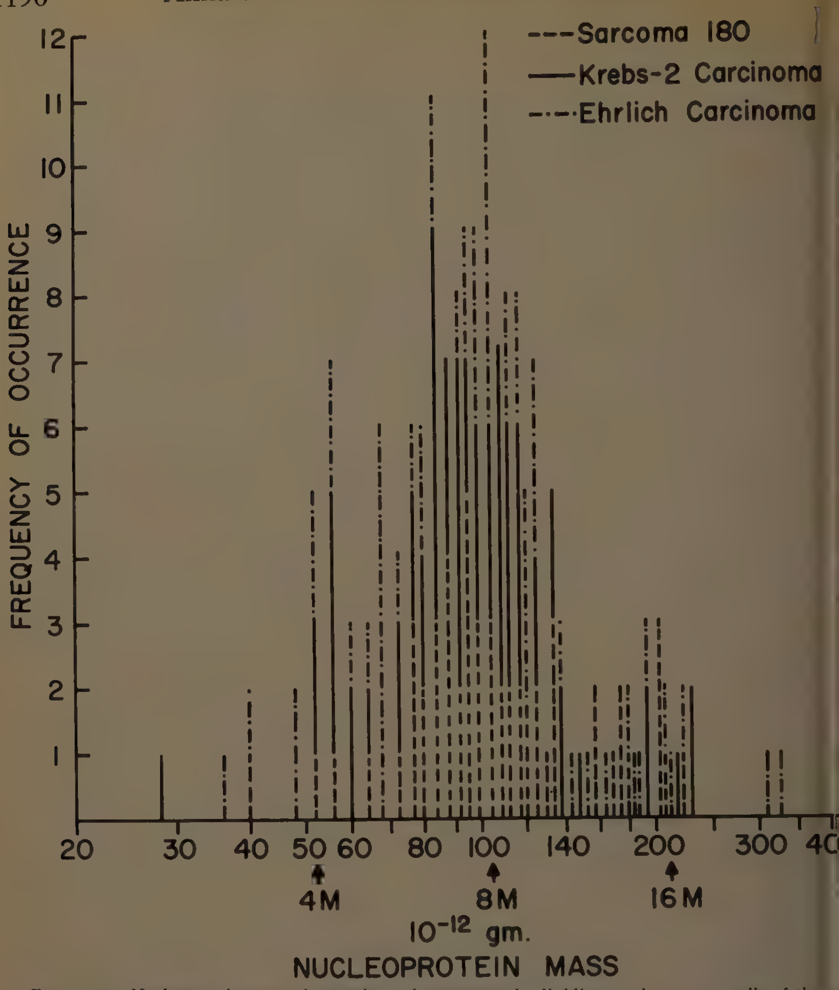

FIGURE 11. Nucleoprotein mass of metaphase chromosomes in dividing, ascites tumor cells of the m (sarcoma 180, Krebs-2 carcinoma, and Ehrlich carcinoma) as measured by microinterferometry. The most mon values center about the arrow at 8M (that is, at about eight times the nucleoprotein mass of the sperm cleus), while lesser modes are present at 4M and 16M. These masses reflect the influence of the number an size of the chromosomes in these cancer cells which are known to be polyploid or polytene.

nizing the complex engineering aspects of the problem, the collaboration of industrial electronics research organization was obtained. The developme work has been continued by this organization, recently at an accelerated r as a consequence of which quantitative data on a large statistical scale wil acquired and the practical feasibility of manufacturing an instrument, ca a Cytoanalyzer, will be determined.

(*b*) *Cytoplasm.* Recent advances in electron microscopy reveal the c acteristic ultrastructure of cytoplasmic components: the mitochondria, with a mean diameter in least dimension of about 200 $m\mu$; the endoplas reticulum,[76] 50–150 $m\mu$ in diameter and more or less equivalent to the l

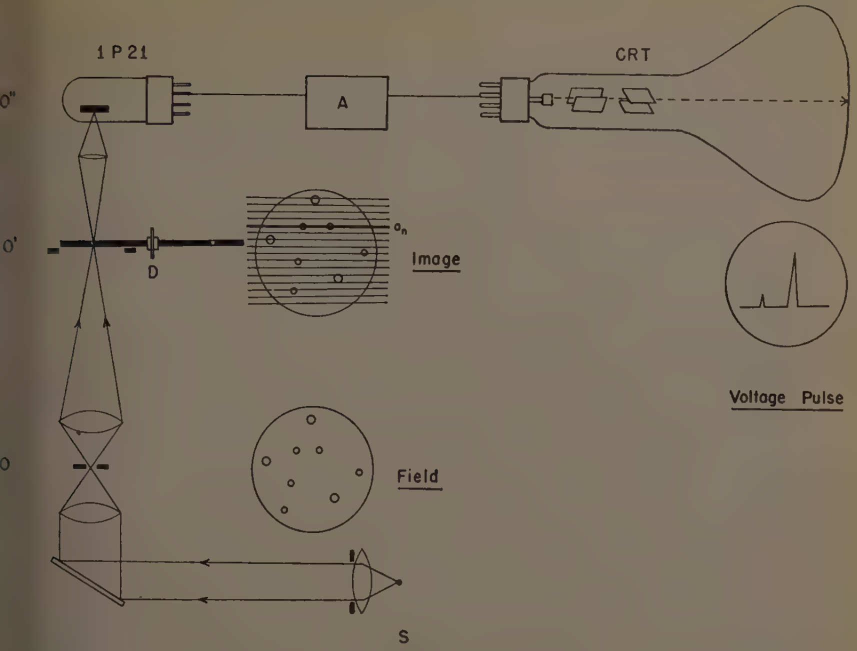

FIGURE 12. Diagram of a scanning microscope which converts optical events in the field or the image plane the microscope into simultaneous electrical events (voltage pulses), thus providing a physical means for the fferential detection of cells that is potentially applicable to exfoliative cytology. From Mellors and Silver.[55]

icrosome component in homogenates; and the small particulate compo-ents,[69] 10–30 mμ in diameter, which apparently are responsible for the diffuse ytoplasmic basophilia of rapidly growing normal or malignant cells coin-iding in size with that of macromolecular components of the cytoplasm studied y Petermann and associates.[71] These small components are found to contain s much as 50 per cent nucleic acid by weight. An additional cytoplasmic omponent with an average diameter of about 120 mμ and designated variously s growth granule,[77] ultrachondriome,[66] and osmiophilic granule[82] has been und with frequency in *in vitro* cultures of cancer cells of the mouse, the rat, nd man, although the full characterization of this component has not yet een achieved. Limitations of space do not permit a further discussion of the toplasm which, quite as well as the nucleus itself, may contain the vital clue the origin of the cancer cell. A scholarly exposition of the many biochemical pproaches to the cancer problem is found in the excellent monograph by reenstein.[26]

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EVALUATION OF THE COLOR-TRANSLATING ULTRAVIOLET MICROSCOPE*

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Ultraviolet as a light source in microscopy has been utilized for a good ma years. Caspersson,[1] in his classical monograph published in 1936, gave major impetus to this new kind of microscopy. The fact that certain biolo compounds absorb ultraviolet light at specific wave lengths opened an enti new approach to the study of biological material.

To determine absorption patterns for certain substances present in ce however, it has been necessary actually to do absorption curves. This i time-consuming and laborious process. For those not familiar with process, it means taking a number of pictures at different wave lengths. photography must be carefully controlled and standardized; *i.e.*, the exposu must be such that background density of the film be as nearly identical possible. The exposures will vary according to wave length, since some ba of the ultraviolet are much brighter than others, and film sensitivity and f gamma vary with wave length. After correct exposure of the film, the velopment also must be carefully controlled, so that results are reproduci The investigator now has a series of negatives taken at different wave leng in the ultraviolet. The procedure to this point must be used in any ult violet microscopy. It is obvious that the accuracy of the results obtai depends entirely on correct exposures and carefully controlled developing.

To determine the absorption curve of the material, we now compare incident light the specimen receives to the amount of light absorbed by specimen at each wave length. This requires densitometer readings. human eye is not particularly adept in distinguishing differences in the sha of gray present on a photographic negative. Because of this, we must res to the densitometer to distinguish whether there is more absorption at wave length than another. Correction must also be made for film gam at each wave length. This process is extremely time-consuming, though accurate curve for a few points in the specimen can be determined. Ma point out also that it is the *shape* of the curve that is important, *not* the *he* of the curve. The shape of the curve depends on *what* material is prese The height of the curve depends on *how much* is present. To know *how m* is present, we must know the thickness of the specimen or cells we are pho graphing. As yet, we have no really reliable method of measuring thickn with the precision necessary for definite quantitative data. It may be, w such new methods as interference microscopy, that we shall be able to det mine accurately the thickness but, up to the present, there is no method general use.

We are faced, then, with something of a problem. Is it worth while to through a laborious process using densitometer readings to acquire information that is largely qualitative and, at best, only roughly quantitative? The answer is, of course, yes, because it is of real importance to know what material is present in biologic material, both in the normal and in disease. A simpler way to acquire such information, however, would be of real advantage.

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* This work has been supported by the American Cancer Society, New York, N. Y.

Land,[2] of the Polaroid Corporation, Cambridge, Mass., has devised a simpler method. It was his concept that, though the human eye can distinguish between shades of gray only with difficulty, there is no difficulty in distinguishing shades of color. If the black and white *negatives* could be translated into color pictures, much of the difficulty in distinguishing differences would be resolved. But, more than a single color would be needed. As much difficulty would be encountered in distinguishing shades of pure red as in distinguishing shades of gray. Therefore, combinations of colors are needed. If this is to be done, then combinations of pictures would be also needed. Therefore, the Polaroid color translating ultraviolet microscope* takes three pictures in rapid succession at three different wave lengths. They are developed, fixed, and dried automatically and, immediately, all three pictures are projected simultaneously on a screen, one picture through a red filter, one through a blue filter, and the other through a green filter. The filters are carefully matched, so that even light coming through all three makes white light. A cell that has marked absorption of ultraviolet light at all three wave lengths will appear white.

FIGURE 1 illustrates such a cell. This is an epithelial cell showing radiation effect, photographed at 240, 261, 280 mμ. Though this picture appears as a single one, remember it is actually three pictures accurately superimposed. FIGURE 2 shows the three pictures out of registration. The upper picture is blue, the middle green, and the bottom red, as can be seen by the pure colors in the tail of this "mouse." We have five separate colors here: pure blue; cyan, a mixture of equal green and blue; white; yellow, a mixture of equal green and red; and pure red. FIGURE 3 shows the same pictures out of registration, but the filters have been reversed, so that red is now in the center. This is to show the third combination of equal mixtures of color; in this instance, red and blue making magenta. These, of course, are examples of two equal color mixtures. In practice, three colors are present, and they are seldom in equal intensity, so that an endless number of variations may be seen. Daily, we take advantage of this kind of color difference. The subtle change in color of an almost ripe tomato to one that is really ripe is an example of changes in mixtures of colors. The color-translating microscope takes advantage of such color differences only to give reliable information about ultraviolet absorption readily and accurately.

Though we mentioned that Land simplified the acquiring of knowledge concerning ultraviolet absorption for the investigator, the machine itself is not simple. It is a complex machine with any number of electric and electronic controls.

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* Developed through funds from the Office of Naval Research, Department of the Navy, Washington, D. C., and the American Cancer Society, New York, N. Y.

FIGURE 4 is a diagrammatic scheme of the machine. The light source is
mercury arc. The light from the arc is admitted by an electronically con
trolled shutter. It then proceeds to a mirror, and to the grating, which ac
as a monochromator. The grating can be rotated automatically, and it
this shift that determines which band of ultraviolet will be transmitted. Eac
of the three pictures may be taken at any desired wave length, and the bar
width may be varied by varying the width of the entrance slit. The ligh
then is sent forward by a series of mirrors. Built into the system is a bea
splitter. This directs 5 per cent of the light to a photoelectric cell in the aut
matic exposure control system. As mentioned before, exposures must
very carefully timed in ultraviolet microscopy in order that all pictures ha
the same background density. The light enters the microscope, as in
ordinary one, through the condenser, through the specimen, objective a
ocular, and is then carried to the film. The objective and condenser are
pecially designed reflecting and refracting lenses[3] that are apochromatic throu
the visible and ultraviolet. The three pictures are taken automaticall
processed automatically, and the negatives projected simultaneously on t
screen through separate color filters.

It is fairly obvious, with even this brief résumé of the machine (FIGURE 5
that it is extremely complex. The investigator is dependent to a great degr
on the ability and understanding of the person who operates it. Any numb
of small details must be accounted for. For example, the exposures must
balanced. The time of exposure of a picture at 240 $m\mu$ may be 10 tim
that for 280. Exposure times will vary according to the age of the arc.
actual practice, an hour or so is spent in getting the machine into operati
for the day. A carbon slide is inserted so that accurate focus can be o
tained and the lenses properly aligned. Then photographs are taken at
wave lengths required for that day. The background densities are dete
mined by densitometer readings and matched to 0.1 density unit by adju
ing the automatic exposure controls. In essence, the use of this machi
requires that all factors be carefully controlled to enable reproducible bac
ground densities at the screen. Only in this way can one be sure that actu
differences are in absorption. As in any precision instrument, it is essent
that all parts be meticulously clean. Stray light tests should be made to che
on the cleanliness of mirrors and grating. Projector bulbs must be proper
lined up. If the picture is red, one must be sure that it is due to absorpti
rather than to a more brilliant bulb. Perhaps this all sounds too elementa
to be mentioned, but it is our impression that the difficulty with some of t
new machines reported from various places throughout the country is exact
due to these factors, which are not new or different. Any good microphoto
raphy requires the same essentials. The color-translating ultraviolet micr
scope is an excellent instrument with a great number of automatic contr
but, in spite of the fact that red and green lights flash in rapid succession a
imply great precision, the operator is still required to be sure these contr
are set correctly and are functioning properly.

We have used this machine primarily to study vaginal smears from patie

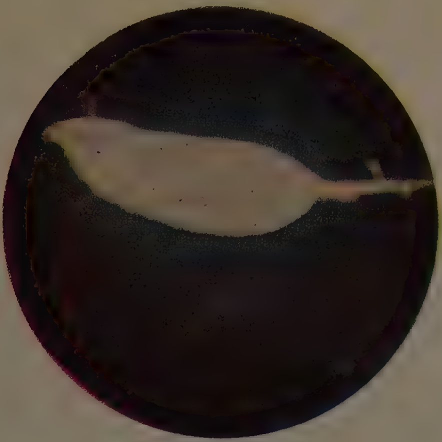


Fig. 1

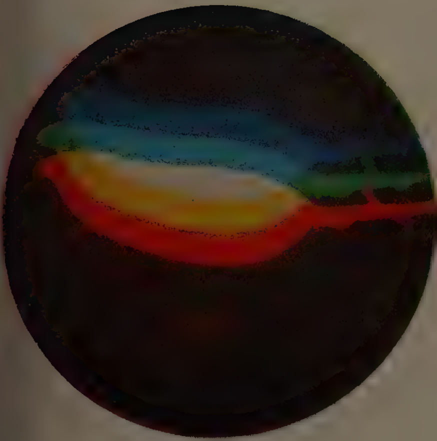


Fig. 2

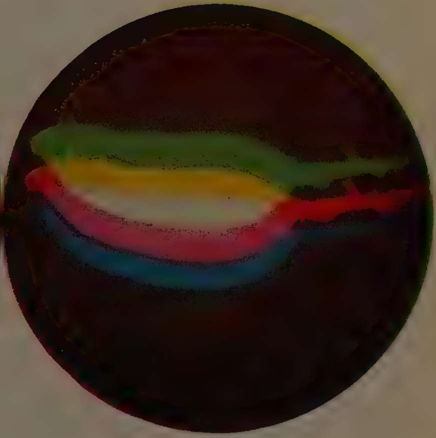


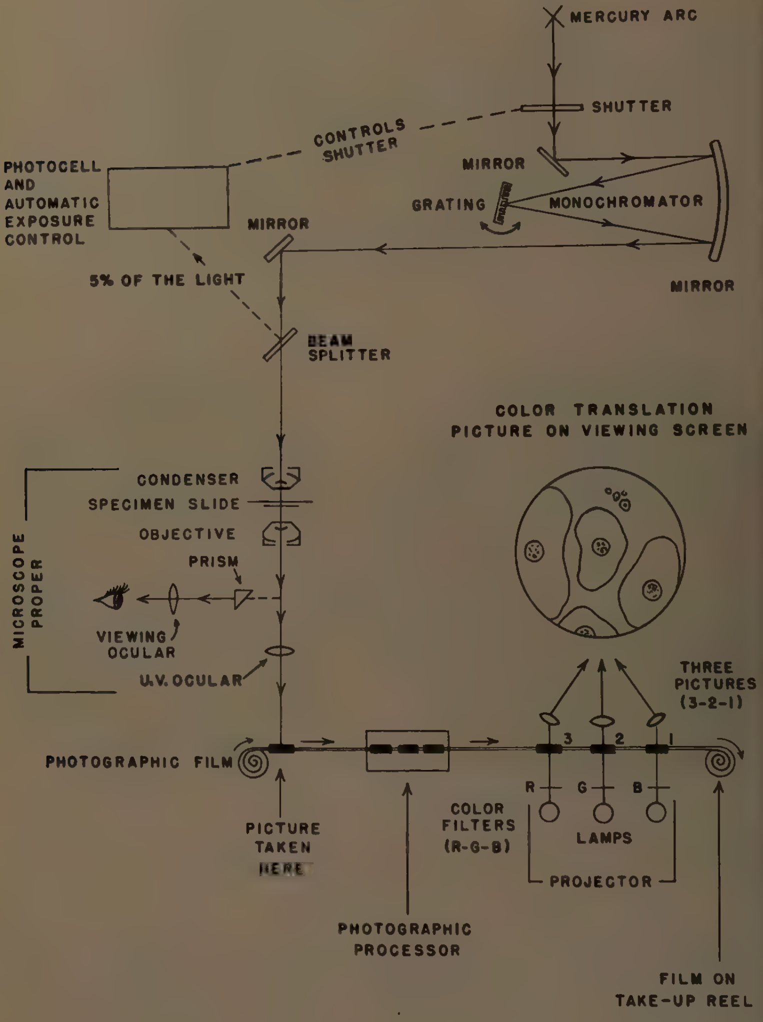
Fig. 3

Figure 1. Radiated benign cell photographed at 240, 261, 280 $m\mu$.
Figure 2. Color pictures in figure 1 out of registration to indicate three separate color filters and combination of colors.
Figure 3. Color pictures in figure 1 out of registration and reversed to indicate additional color combination of equal red and blue = magenta.

with cancer of the cervix. These smears have been prepared in the usual way. The secretion from the posterior fornix was aspirated and placed on a Vycor slide instead of on an ordinary glass slide. This was fixed in a solution of half ether and half alcohol. It is perfectly possible, of course, to use fresh material. We have preferred, however, to use fixed material since, in the fresh mount, cells float about and specific cells are more difficult to find. Each slide is mounted with double-distilled glycerin and a Vycor cover slip. It has been our practice to examine the slide under the phase microscope and

mark the cells to be examined under the ultraviolet. This saves time at
machine.

First let us look at the normal cells of the vaginal smear. The three ba
cells shown in FIGURE 6 were photographed at 280, 261, 240 mμ. The pict



SCHEMATIC DIAGRAM OF THE
POLAROID COLOR-TRANSLATING ULTRAVIOLET MICROSCOPE

FIGURE 4. Schematic drawing of Polaroid color-translating ultraviolet microscope.

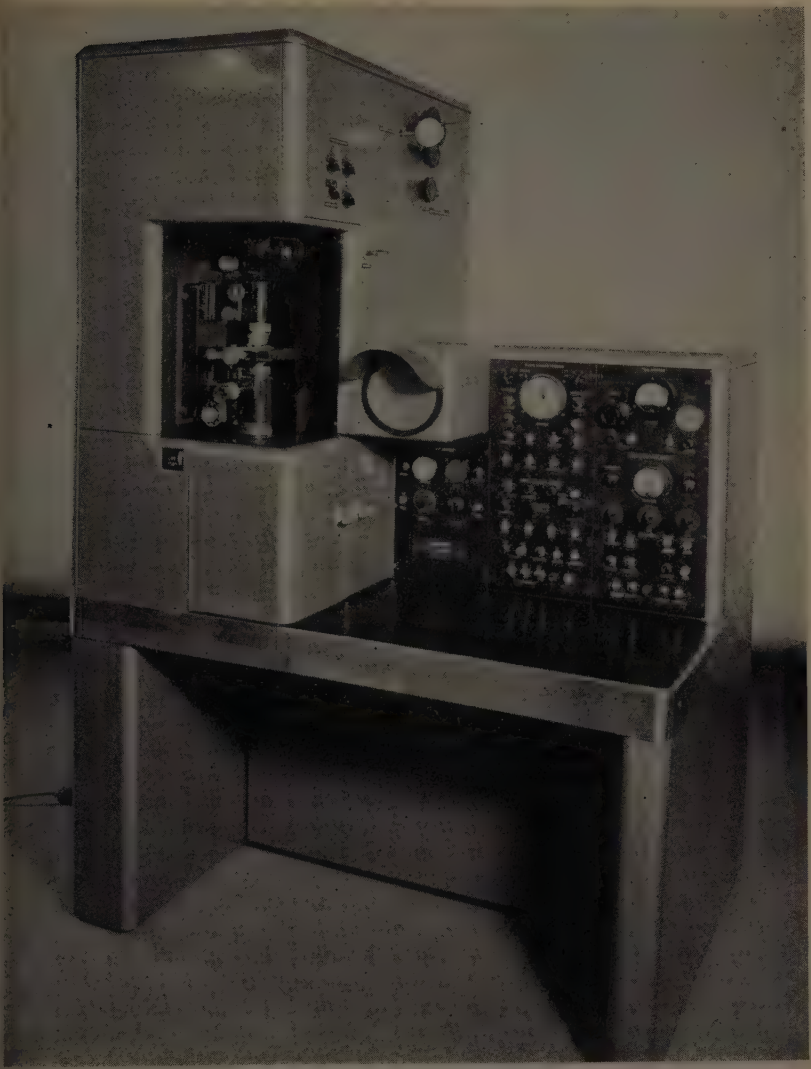


FIGURE 5. Photograph of actual instrument.

aken at 280 is projected through the blue filter, that taken at 261 mμ through he green filter, and that taken at 240 through the red filter. The cytoplasm of these cells is predominantly red, indicating peak absorption at 240 mμ and very little cytoplasmic absorption at either 261 mμ or 280 mμ. The nuclei appear white, however, indicating absorption at all three wave lengths.

In FIGURE 7, two of the previous wave lengths have been shifted—240 to 248 mμ (red filter) and 280 to 289 mμ (blue filter). The middle wave length projected through the green filter remains at 261 mμ. It is obvious that there

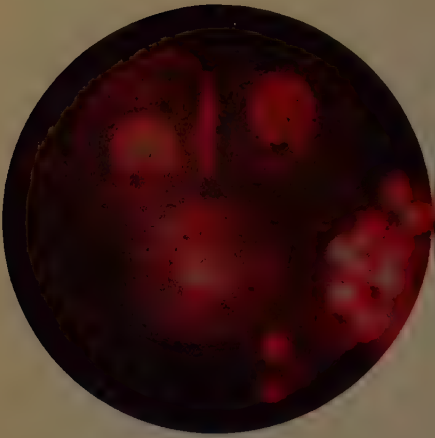


Fig. 6

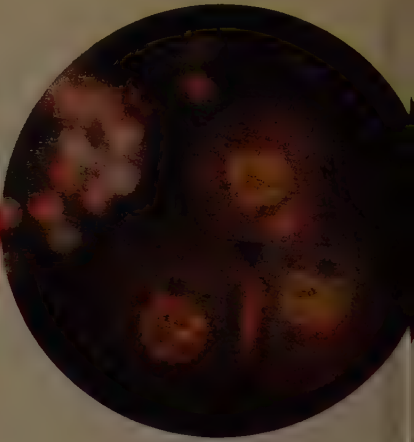


Fig. 7

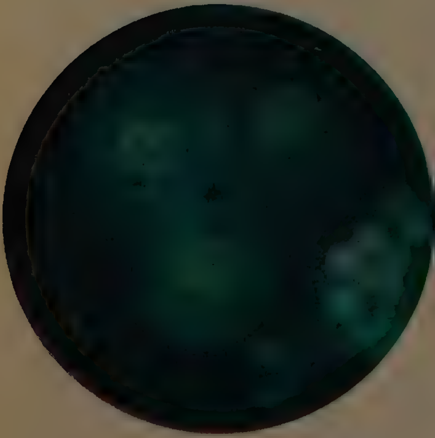


Fig. 8

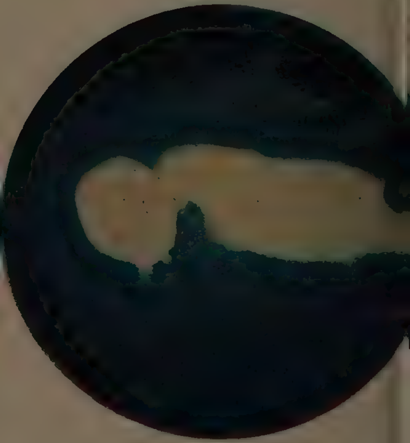


Fig. 9

Figure 6. Normal basal cells from vaginal smear photographed at 280, 261, 240 mμ.
Figure 7. Identical field as figure 6 photographed at 289, 261, 248 mμ.
Figure 8. Same field as figure 6 photographed at 297, 261, 302 mμ.
Figure 9. Two benign cells showing radiation effect photographed at normal exposure in the ultraviol
240, 261, 280 mμ.

is a marked color change in the cytoplasm from the pronounced red seer figure 6 to very little definite color. This indicates that the curve for cy plasmic absorption drops very rapidly between 240 and 248 mμ. The nu have also changed in color to a yellow. This indicates almost even absorpt at 248 and 261 mμ with no absorption at 289 mμ.

If the wave lengths are shifted to even higher wave lengths, a picture s as shown in figure 8 will be obtained. In this instance, the 289 wave b has been shifted to 297 mμ (blue filter) and the 248 band to 302 (red filt The negative projected through the green filter remains the same, having b

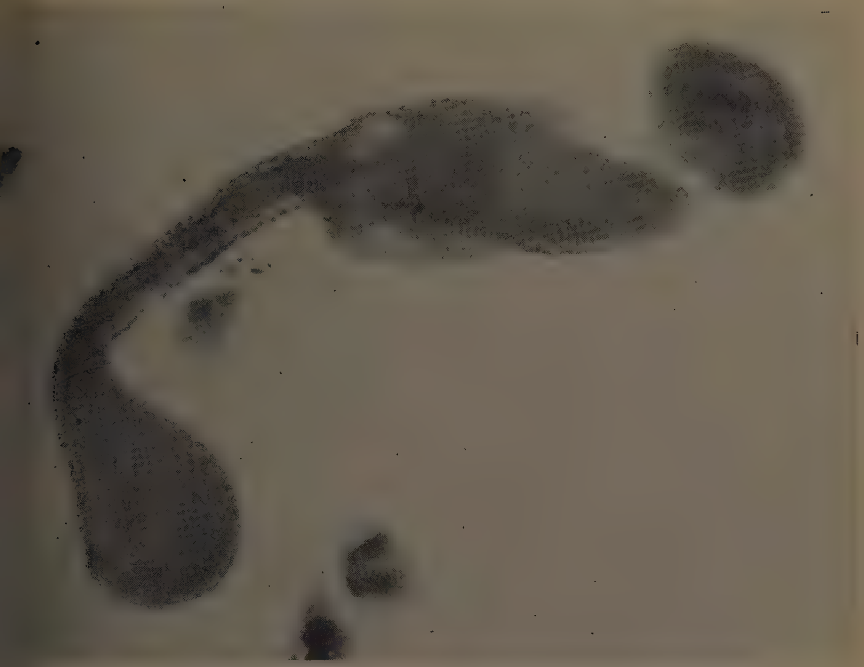


FIGURE 10. Identical cells as in FIGURE 9, stained by Papanicolaou's technique.

hotographed at 261 mμ. It can readily be seen that there is no absorption at ny wave length except 261 mμ, since no color except green appears.

After such a series of pictures as these, it is possible to plot a rough absorp- on pattern for both the cytoplasm and nuclei, since the absorption at different ave lengths may be compared one with the other.

An example has already been shown of the marked absorptions seen in me cells showing radiation response. For another example see FIGURE 9. hese photomicrographs were taken at 240, 261, and 280 mμ at normal ex- osure. We have a white cell, which means tremendous absorption at all ave lengths. Such a cell, if stained in an ordinary way, will not look dis- nctive, as might be expected. FIGURE 10 shows the same cell after the glycerin as been removed and it has been stained by the usual Papanicolaou tech- ique. We have a single, fairly normal-looking basal cell and, to the right, a diated basal cell. Nuclei are discrete though, in the ultraviolet, the cyto- lasmic absorption was so intense that no nuclei could be seen. This cell serves an example of the stimulating type of information that can be discovered by se of ultraviolet microscopy.

In summary, the color-translating ultraviolet microscope has several ad- ntages over ordinary ultraviolet microscopes:

First, it is possible to determine qualitative absorption for three different ave lengths at a glance, because of color differences.

Second, the *total* absorption of the material may be investigated, not ju a series of points, as in absorption curve data.

Third, the speed of operation is an important factor, as only a few minu are required to complete the entire operation.

The microscope's sole disadvantage is that it is a complex machine and th it requires exact supervision. To a large extent, the results obtained will pend on the proficiency and understanding of the technical personnel who the machine for the biologic investigator.

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AUTOMATIC SCREENING OF CYTOLOGICAL SMEARS FOR CANCER: THE INSTRUMENTATION*

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Introduction

Mass screening for the early detection of cancer, by the techniques of exfoliative cytology, may become a reality if automatic aids can be brought to bear that can increase significantly the number of smears that can be processed by cytology staffs and facilities. We are developing a key instrument toward this objective: the cytoanalyzer, a high-speed automatic device to eliminate the need for human effort now consumed in screening distinctly normal smears. This current development is an outgrowth of an earlier proposal by Mellors, Glassman, and Papanicolaou.[1]

Design Basis

The basis for such a device is found in quantitative differences between normal and abnormal cells of conventional Papanicolaou smears. A study of vaginal and cervical smears of a 250-patient group has been reported in part.[2] This study showed, by measuring the nucleus diameter, nucleus density, and cell diameter, that clear-cut differences existed between normal and abnormal smears.

With nucleus density plotted against nucleus diameter, each scanned cell in a sample becomes a point on a graph. The study showed that, for most negative smears, these points fell within a well-defined area, that for positive smears and for a small percentage of negative smears, a large number of cells —up to 20 per cent—fell outside the boundary because they were abnormal in diameter, or density, or both.

Cells aberrant in nucleus density and nucleus diameter also possessed uniformly small values of the cytoplasm/nucleus ratio compared with that for normal cells. Whether this quantity will need to be exploited in the cytoanalyzer is still undetermined.

Functional Design

Functionally, the cytoanalyzer (FIGURE 1) comprises three major units: scanner, analyzer, and recorder. The general scheme of the analyzer (measurement and control circuits) has been reported.[3] This paper will report in some detail the scanning unit, because this unit is fundamental to high-speed microphotometry, is of special interest and use to quantitative cytologists, and has achieved a quite satisfactory status of performance. Discussion of the other units will be limted to a brief explanation of their functions.

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* This project is supported in part by a special purpose research grant from the American Cancer Society, New York, N. Y., and in part by a field investigation grant from the National Cancer Institute, National Institutes of Health, United States Public Health Service, Bethesda, Md.

† The authors are indebted to the Strang Laboratory of Cytology, Memorial Center for Cancer and Allied Diseases, New York, N. Y., for the loan of slides used to prepare the photomicrographs accompanying this paper.

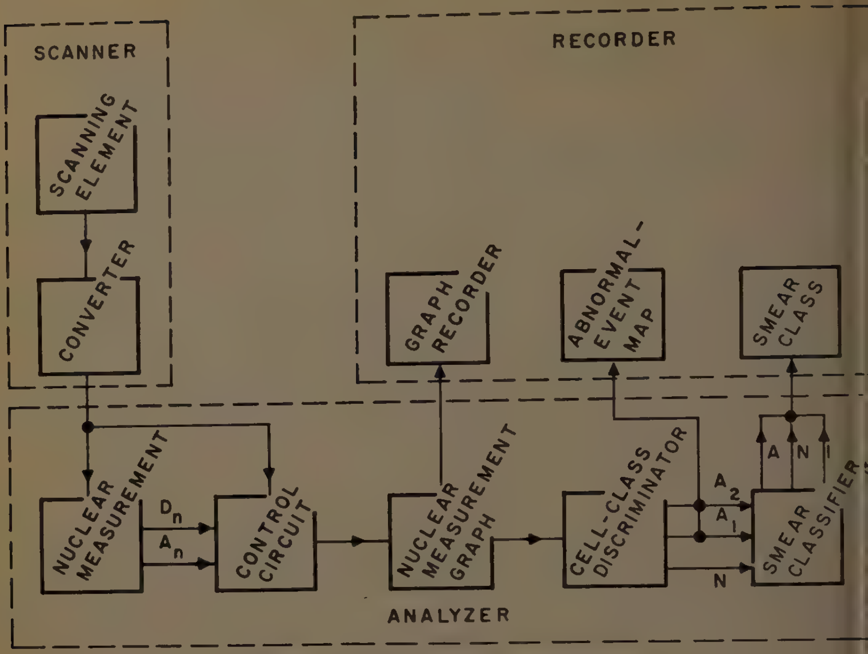

FIGURE 1. Function diagram of cytoanalyzer.

The scanner examines the pertinent area of the smear with respect to a
sorbing objects, and converts the optical information about such objects—
terms of density and space occupied—into electrical analogues: amplitu
and time. Signals from the scanner enter simultaneously (1) the measureme
circuit, where the measured values are stored for later plotting upon approv
by the control circuit; and (2) the control circuit, where plainly false mea
urements—those produced by clusters of overlapping cells—are rejecte
Each accepted measurement is plotted to permit the cell-class discriminator
rate it as normal or abnormal. On the basis of the distribution of all the mea
urements from a smear, the smear is classified as: (1) normal; (2) abnormal;
(3) deficient in required information. The recorder makes a permanent reco
of the nucleus-dimension graph, notes on a remote map of the slide the lo
tion of all abnormal measurements, and records the decision of the sme
classifier.

The Scanner

1. Resolution. A primary factor in the design of a scanner is the cho
of resolution, defined as the reciprocal of the minimum separation of two o
jects that will provide a discrete signal from each. It is obvious that the su
ject material of interest to the investigator will itself determine the resoluti
that will be required. To scan for particles as small as mitochondria wou
require resolution of the order of ½ to 1 μ; for the lobes of polymorphonucle
leucocytes, 1 to 2 μ; and for pyknotic nuclei, 2 to 4 μ. As we are principal

concerned here with nuclei ranging from 5 to 30 μ, we have chosen 5 μ as the resolution of the scanning system.

By this is meant that the field stop through which the object-plane material is viewed is, in effect, a circular hole of 5-μ diameter. Any object smaller than this size will fail to fill the sample area, and will therefore give severely reduced values of density. Thus, the sample area acts as a mechanical filter tending to ignore objects smaller than itself. Objects larger than 5 μ can be measured accurately in size and density, and density differences due to variable granularity are averaged over this generous sample area.

It is evident that the size of the sample area controls the flux of energy to the detector. It is advantageous to use as much light as possible, consistent with resolution requirements. Thus, the use of an unnecessarily high resolution would reduce the speed of scanning and introduce data not pertinent to the problem at hand.

A mechanical scanner is used with the cytoanalyzer. This means that the resolution must be obtained by a physical field stop in the system that will give a stop of the required diameter in the object plane. To form uniform accurate holes with a 5-μ diameter is highly impractical, if not impossible. Therefore, optical magnification is employed to allow a practical hole diameter to act as a 5-μ aperture. A magnification of ×45 is used, which permits the actual stop in the image plane to be slightly over 0.2 mm. diameter, or 0.008 inch, a hole easily realized either by drilling or etching.

Figure 2 is a schematic diagram of the scanner. Conventional microscope optics was used when possible. The ×45 magnification of the scanning field is obtained with a 4-mm. achromat objective. Placing the field stop in the image plane of the objective permits the use of full field illumination, making

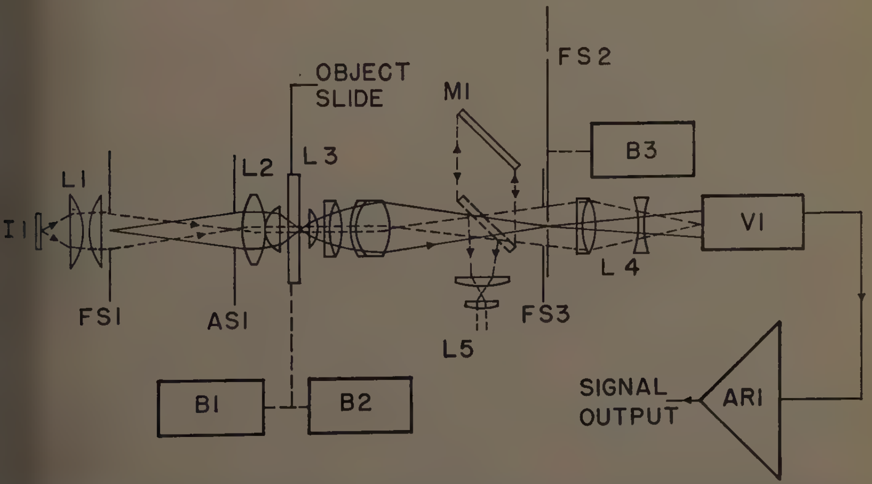

Figure 2. Schematic diagram of scanner unit. I1, tungsten lamp. L1, lamp condenser lens. L2, substage condenser lens. L3, objective lens. L4, collector lens. L5, monitoring eyepiece. AS1, substage aperture stop. FS1, illuminator field stop. FS2, scan disk. FS3, fixed field stop. V1, photomultiplier. M1, sliding mirror. AR1, preamplifier. B1, scan advance stage drive. B2, raster advance stage drive. B3, scan disk motor.

it possible to check the operation by visual observation of the scan ar through a monitoring eyepiece. A conjugate system, placing the apertu in the source plane, would yield a flying-spot scanner, perhaps better for resol tion and contrast, but inconvenient at the present stage of development.

Uniform field illumination is essential if accurate absorption measuremen are to be made at every field position. To achieve optimum alignment of t Köhler illumination system, precise centering and focusing adjustments a provided for the condenser lenses and the light source—a ribbon-filame tungsten lamp. To eliminate cyclic variations in intensity, the lamp is ope ated from a filtered direct-current supply. Limiting the field to 250 μ wi a fixed field stop in the image plane limits to less than 2 per cent the vari tions in the illumination of the scan aperture as it is moved across the fie

The light passing through the scanning field stop falls on the cathode of t photomultiplier tube, which converts the light intensity into a proportion electric current. An end-on, 11-stage, 6467-type tube is used because it h a large, flat cathode area and high cathode-current rating. For these pu poses, the tube is quite superior to a small curved-cathode tube like the 1P obviating the need for critical alignment and accommodation to grid w interference. The collector lens directly behind the scan disk is used to p vent image movement on the cathode, which itself has a nonuniform spat sensitivity, by focusing the image of the stationary lamp filament on t cathode surface.

FIGURE 2 shows clearly the complete symmetry of the optical elemen about a center line defined by the object plane. Each element on one side the object plane has a physical conjugate on the other side. Such symmet simplifies the understanding and procedures of alignment necessary to hi resolution, good contrast, and uniform illumination.

The photomultiplier is connected to a direct-coupled preamplifier who output is applied to the analyzer unit. The preamplifier limits the ba width to 250 kilocycles per second, has a gain control for adjusting the sign output level, and provides a low output impedance for coupling to the analyz

FIGURE 3 shows the scanning pattern. The scanning field stop is plac on the scanning disk in the form of 30 uniform holes evenly spaced on a mm. radius. As a hole passes over the 250-μ fixed field stop, one line of sc is produced. The actual scan line generated is an arc of a circle, causing departure from a straight line of only 1 part in 50, unimportant to the inte rity of the scanning process. Because the spacing between the holes is grea than the 250-μ width of the scanned field, there is a black, or no-curre period between each two lines of scan, a reference useful in signal-level a justment and in control of analyzer functions.

The disk rotates at a constant speed of 30 revolutions per second, there generating 900 scan lines per second. Simultaneously with the rotation the scan disk, the slide is advanced forward at a uniform velocity in a directi perpendicular to the scan line. The scan advance is slightly less than t width of one scan line, to give a small amount of overlap (for clarity, in t illustration it is made to appear somewhat greater than the width of one sc

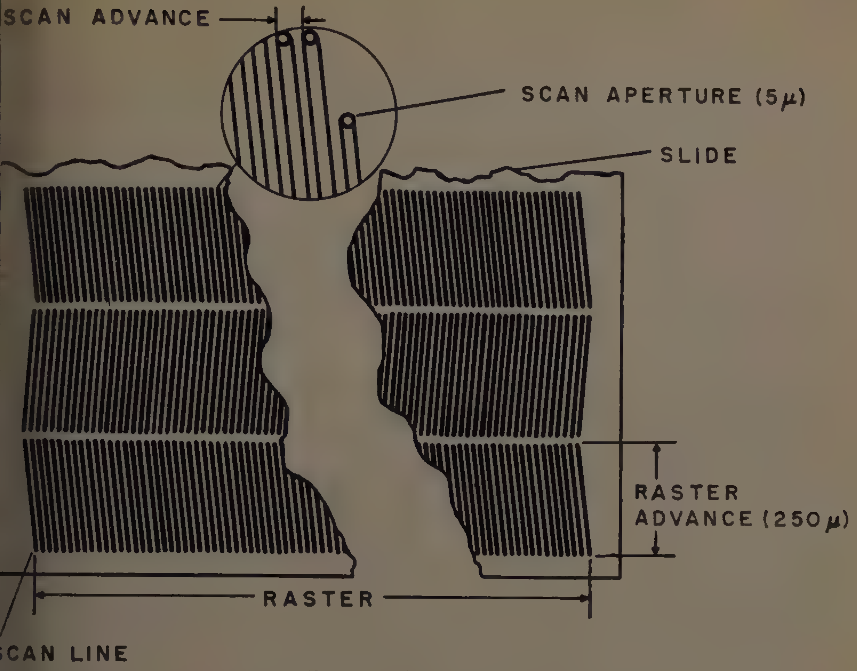

FIGURE 3. Scanning pattern.

ne). One raster is produced by advancing the slide the length of the portion ccupied by the smear. When the scan has advanced the length of the smear, he slide is moved 250 μ in a direction perpendicular to that of the scan ad- ance. The direction of scan advance is then reversed, and a second raster s produced parallel to the first. This pattern progresses until the entire slide s scanned.

A difficulty associated with scanning a large area of the slide is maintaining onstant focus. The main sources of focusing error were found to be warping f the slide and vertical displacements of the mechanical stage. To eliminate hese errors, spring pressure is applied to the slide to keep it in contact with a ripod of short base formed by three ball points closely spaced around the ondenser to form a plane of constant focus. The depth of focus is suffi- ient to keep within reasonable bounds the errors that result from normal ariations in thickness of slide, cover glass, and specimen.

2. *Scan velocity.* With the choice of resolution made, the second funda- ental factor in the scanner design is the choice of scan velocity. In the ectrical signal containing nucleus size and density information, two objec- ves must be met: the transformation from image to signal must be faithful nough to allow good measurements to be made on pulse height and width, nd the noise constituents of the signal must be small enough to give a high egree of reproducibility of a repeated measurement. The signal-to-noise haracteristic of the photoelectric detector determines the dual choice of the

two design parameters: frequency band width (or velocity), and the fina adopted signal-to-noise ratio.

At high light levels, photomultiplier dark-current noise is negligible, a the shot noise becomes the principal source of noise. Under this conditi the signal-to-noise ratio will be proportional to the square root of the lig intensity, and inversely proportional to the square root of the frequen band width. The band width is directly proportional to the scan veloci Therefore, for a given signal-to-noise ratio and degree of signal fidelity, t scan velocity will be proportional to the light intensity.

The scan velocity used on the present model of the cytoanalyzer is 3×1 μ per second in the object plane. With a 5-μ resolution and a band width about 250 kilocycles per second, the signal attenuation is less than 2 per ce with the filter used in the present model.

With the same resolution, and with no absorbing object on the slide, t light intensity measured on the cathode is 15 microlumens. Under the conditions, the signal-to-noise ratio produces an average uncertainty of t amplitude of only 2 per cent. This scan velocity, plus the dynamic geom rical constants of the scanner, gives a total reading time of four minutes fo smear area of 1 cm. by 2 cm.

It is estimated that, in the design of a clinical model, the reading spe can be increased by a factor of about 10 by increasing the light intensi using sharper filters, and accepting a slightly reduced signal-to-noise rat

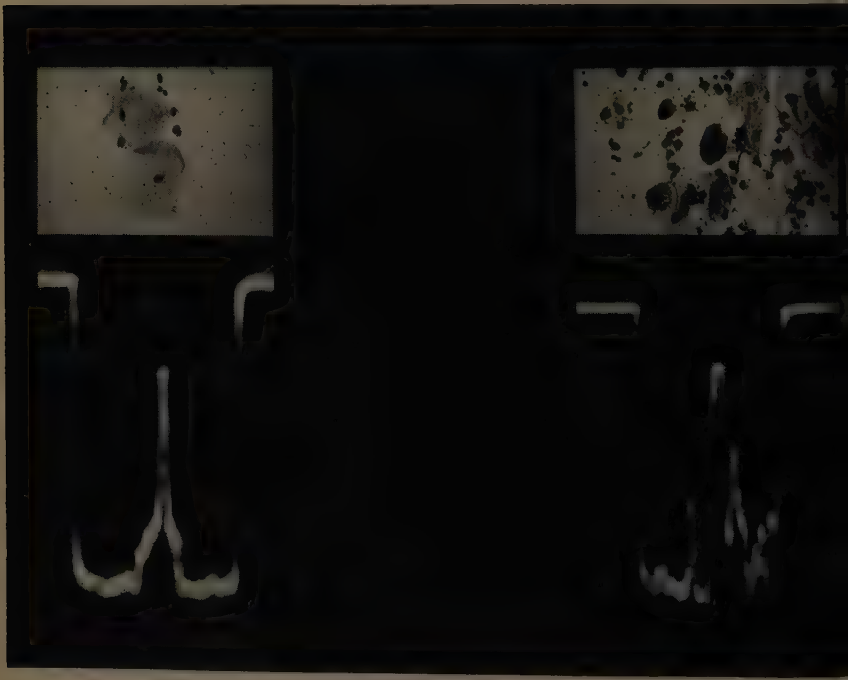

FIGURE 4. Output signals from normal (left) and malignant (right) cells.

Results

FIGURE 4 shows, at the left, the preamplifier output signal from one horiontal line of scan through the nucleus of the basophilic normal cell. Although he photomicrograph and oscillograph are only approximately to the same cale, it is readily apparent that the nucleus diameter is proportional to the ulse duration of the narrow spike, and that the peak pulse amplitude is proortional to the light absorption of the nucleus. The same relations can be bserved for the cytoplasm.

At the right, in FIGURE 4, is the corresponding signal from a malignant cell. his larger and denser cell gives a pulse of correspondingly greater duration nd amplitude.

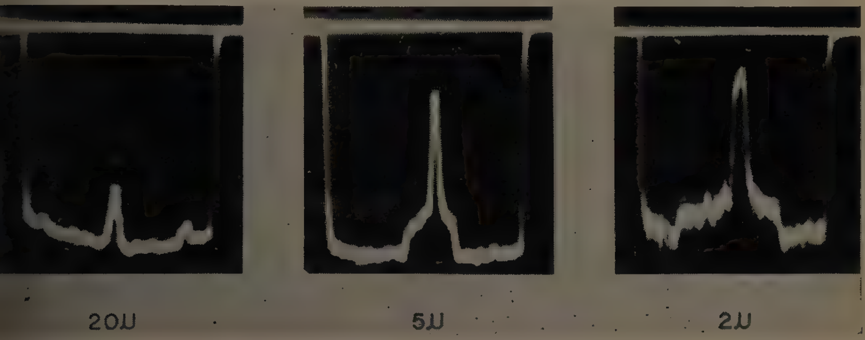

FIGURE 5. Effect of change in resolution.

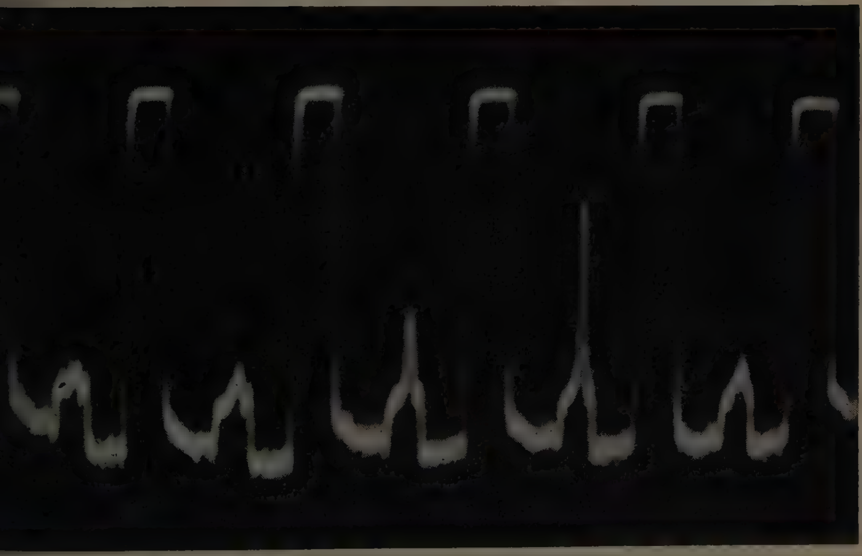

FIGURE 6. Output signal from successive scans.

The effect of resolution on signal output can be seen in FIGURE 5, where normal cell already shown in the preceding figure is scanned successively w apertures of 20, 5, and 2 μ. The 20-μ aperture, being many times as la as the diameter of the nucleus, exhibits only the cytoplasmic density, nuclear detail being lost. The 5-μ aperture, being about equal to the nucl diameter, clearly exhibits the cytoplasm pedestal surmounted by a triangu nuclear pulse. The 2-μ aperture shows the most detail, sharp cytoplas boundary, a cytoplasmic inclusion, and a well-defined nuclear pulse.

These traces also show that, for a constant level of field illumination, increase in resolution is obtained at the cost of a lowered signal-to-noise ra

FIGURE 6 shows the signal from this same normal cell from a sequence scan lines, each line representing an advance of about 5 μ. Most of the li indicate pulses from the cytoplasm. One shows a full pulse from scann through the center of a nucleus, another a partial pulse from scanning throu only a segment of a nucleus. Although the partial signals (chords of nucleus) introduce extraneous measurements, such measurements are nec sarily lower in amplitude and shorter in duration than the full-diameter s nals, and hence introduce no error in classifying a cell signal.

Summary

Results from scanning actual smears show that the scanner does relia and accurately transform the cell information on the smear into electri signals that can be analyzed to obtain measurements of nucleus size and d sity. The mechanical image-plane scanner described has high sensitivi good signal-to-noise characteristics, reliable operation, and economical c struction. Its design is flexible, and it can accommodate to numerous pr lems where requirements of subject material, resolution, and illumination m all be different. These qualities make this type of scanner highly satisfacto for this application.

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TOPOLOGICAL CYTOCHEMISTRY

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Topology is a branch of mathematics dealing, *inter alia*, with certain qualita- ive properties of geometric figures that remain invariant even though such onfigurations may undergo drastic deformations.[1,2,3] For example, the onnectivity of surfaces would be the same on deforming a spheroid into either n ellipsoid, a disk, a rod, a polyhedron, or even an irregular mass that, in ddition, may be continuously flattened. On the other hand, a torus cannot e deformed into a spheroid, nor can a spheroid be converted into a torus ithout violating topological rules. Another important topological feature eals with modifications of two- and three-dimensional structures so that hey possess only one surface and one edge. Some of the topological properties f various figures are illustrated in FIGURE 1.

The homeomorphic transformations of cells are illustrated in FIGURE 2. he conversion of initially spheroidal cells to other shapes is a common and enerally spontaneous event that occurs during the development of an or- anism or when cells grow in tissue cultures. Cells frequently become flat- ened or spindle-shaped in tissue culture. The differentiation of cells into ighly dendritic structures is also an example of continuous transformation. As shown in FIGURE 2, a flat cell looks larger than a spheroidal cell despite he fact that both may have the same volume. The changes in shape of uclei also represent homeomorphic transformations. It is important to emember that a cell undergoing a change in shape may do so only as far as the ytoplasm is concerned, whereas the nucleus may not become flattened to the me extent. Thus, nucleo-cytoplasmic ratios as determined from areas ay be misleading. Such ratios should be calculated on the basis of volumes ather than areas.

Topology and Cytochemistry

Cytochemistry in its newer aspects stresses the quantitative determination f chemical constituents in a cell and also the association of enzymes with bcellular particulates or structures. Topological cytochemistry is a field of esearch that attempts to define the properties of subcellular structures in rms of chemical composition, physicochemical or biochemical functions, teractions with other cellular structures, time, and form.

It must be remembered that cytochemistry has definite limitations, at esent, despite advances in estimating small amounts of chemical substances low activities of enzymes. Clear-cut distinctions generally can be demon- rated between RNA or DNA, however, it is impossible to distinguish between rious kinds of DNA or RNA in subcellular structures. To illustrate, one romosome of a homologous pair cannot be distinguished cytochemically om the other despite possible differences in genetic or other biologic poten- als.

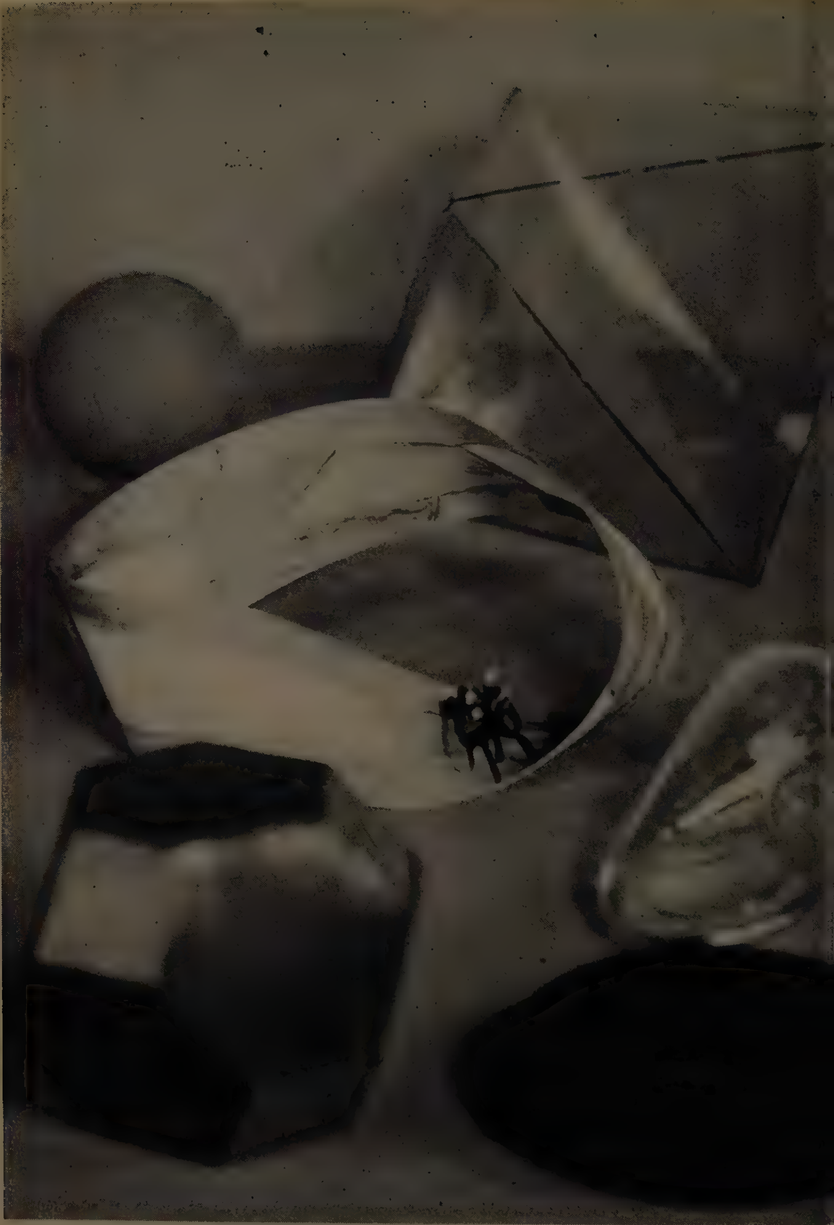

FIGURE 1. General topological properties. The sphere and torus (foreground) exemplify nonhomeomo
structures, since one cannot be transformed continuously into the other. The sphere would have to be pun
or the torus would need to be cemented or cut in order to permit a continuous transformation. On the other
the sphere can be converted into a tetrakaidecahedron, or *vice versa*. The tetrakaidecahedron, a truncated
hedron frequently associated with the shapes of compressed spheres, or cells,[4, 5] is a solid that comes closest
polyhedra to filling space with minimum surface area.[6] The tetrakaidecahedron also satisfies Euler's the
i. e., $V + F = E + 2$ (Vertices + Faces = Edges + 2).[1]

The Moebius strip (center) has only one surface and one edge. The crab shown in the photograph could
over the entire surface without ever leaving the surface. Similarly, a map can be drawn on a Moebius strip
requires a minimum of six colors (no two regions with common boundary to receive the same color), wh
maps so far prepared require only four colors if on a plane surface. The Klein bottle also has one surface
has no *inside* or *outside*. If bisected sagittally, it would form two Moebius strips. The transparent, mo
octahedron (upper right) has only one surface and one edge. This three-dimensional figure, therefore, ha
same properties as a Moebius strip.

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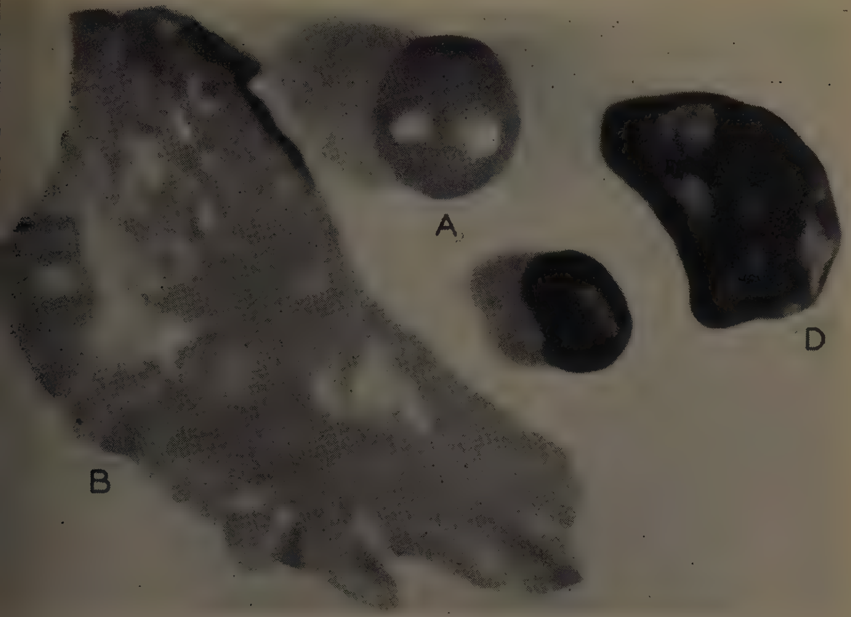

FIGURE 2. Homeomorphic transformations. Spheroidal cells can be transformed continuously into flattened
s, and vice versa. The figures shown are photographs of plastic models of cells growing in tissue cultures
man ovarian neoplasms). The spheroidal cell, *A*, actual diameter *ca.* 30 μ, is the usual shape of such cells cul-
ed in fluid media. The flattened cell, *B*, is seen frequently in solid media. Although the flattened cell looks
ger, its volume is identical to that of the spheroidal cell. Both nuclear models are scaled to the cell models.
smaller nucleus, as seen in the spheroidal cell and the larger nucleus, *D*, seen in the flattened cell, have the
e volumes.
This group illustrates the plastic nature of cells and nuclei, and it emphasizes the necessity of considering all
e dimensions of both the cell and nucleus. This becomes important in calculating nucleocytoplasmic ratios.

Even though two structures may have identical chemical content, they
y be dissimilar owing to subtle differences in form. For example, the two
foil knots, *A* and *B*, in FIGURE 3, cannot be continuously transformed one
o another, and hence these represent distinctly different structures.[1] This
a clear instance of the diffiulties encountered in developing complete defini-
ns of a structure if the information is based on either its chemical properties
its form alone. If both configurations were microscopic in size and sec-
ned, it would be impossible to distinguish between the two. On the other
nd, several structures could have the same basic forms, yet they would not
identical because of chemical content.

The basic concept, then, is that the properties of any part of a cell cannot
completely defined unless both its chemical composition and structure are
own. Furthermore, it is essential to consider the interaction of these struc-
es with other structures or substances in a cell, together with the probable
luence of genetic constitution.

esearch on the cytochemistry of malignancy should be augmented by direct
erimentation on cells such as procedures for isolating and transplanting
cellular structures. Promising strides have been made in such experimen-

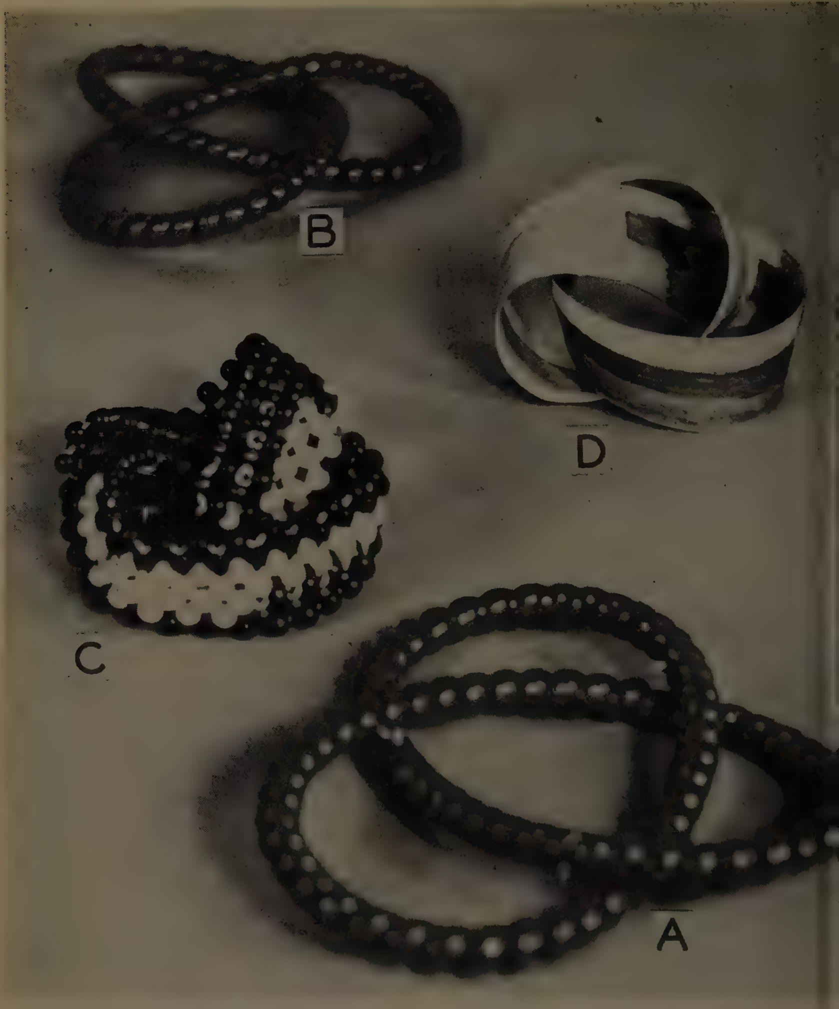

FIGURE 3. Form and "chemical composition". *A* and *B* are examples of trefoil knots that have ider chemical properties, both qualitative and quantitative. These two figures are noncongruent and, hence, ca be transformed into the other continuously. Knots *C* and *D* are topologically identical to *A*, yet it is obvious they are not the same, because they were made of different materials and, hence, their compositions are dissir Both form and chemical composition are required for an adequate definition of the properties of a structur

tation as indicated by recent improvements in qualitative and quantita micrurgical procedures and in maintaining single cells under conditions p mitting their survival, growth, and even reproduction. The methods suita for determining the properties of certain subcellular components by combir topology with cytochemical micrurgy will be discussed in this report.

Determining the Function of Nucleoli

One of the parts of a cell that is least understood, in terms of its functio is the nucleolus, although this structure has been recognized even by the ea ytologists. Some of its chemical and morphologic features are already nown. Moreover, Pianese[7] in 1896 had implicated the nucleolus as an imortant factor in malignancy.

How can one find out exactly what the nucleolus is and what it does? One pproach is through the experimental transfer of the nucleolus to other parts f a cell or even to different cells. Certain reactions between the nucleolar ody and the recipient cell, or part thereof, may thereby be studied and evaluted. Thus, the cytoplasm of the same or different cells can be utilized as a hysicochemical substrate with which the nucleolar body may interact. The irst approximations will hinge on effects produced in the nucleolar body folowing transfer to another site, and also on the action of the nucleolar body n its new environment.

Through the development of cytochemical micrurgy,[8] as well as new proedures for subcellular transplantation,[9] it now seems feasible to attack the ucleolus with the aim of elucidating its role in the biology of the cell. In ddition to the experimental procedures, certain other features have been onsidered, such as the application of calculus of sets[10, 11] and the theory of roups.[12] Symbolic logic can also contribute, if for no other reason than that simplifies the proper design of critical experiments.

Experimental Procedures for Transplantation Studies

During recent years, there have been several interesting and frequently uccessful attempts in executing subcellular transplantation, ranging from ytoplasmic transplantation (Duryee[13]) to nuclear transplantation (Comandon nd de Fonbrune,[14] Lorch and Danielli,[15] Briggs and King[16]). The various ifficulties involved in subcellular transplantation have been extensively tudied and certain improvements in procedure have emerged.

One of the most important requirements for increasing the success of subellular transplantation is the elimination of water from the environment of ells during micrurgy. For this, the fluorocarbon-transfer procedure has een developed (Kopac[9]). Subcellular structures may be brought in direct ontact with certain fluorocarbon fluids without danger of producing lysis or ther drastic changes such as those that almost invariably occur when protolasmic structures are brought in contact with water or various aqueous soluons.

Most cells can be transferred, by centrifugation, to a nontoxic, nonaqueous uorocarbon fluid. Certain fluorocarbon fluids can be used because of their w surface tensions, reasonably high fluidities, extraordinarily poor solvent tion, and other inert characteristics. A beautiful method for the rapid isolaon of the Rous fowl sarcoma agent, using fluorocarbons, has been recently eveloped by Gessler, Bender, and Parkinson.[17]

Another important advance in subcellular transplantation is the volumetric bmicromanipulator (Kopac and Harris,[18] Kopac[19]). The removal and subquent implantation into other cells of known volumes of subcellular subances of the order of 1 $\mu\mu$l. can thereby be accomplished.

The coagulation of cytoplasm and its adherence to the glass walls of a micropette can be avoided in most instances by first coating the micropipettes with a thin silicone film.[19] By preventing coagulation of the cytoplasm other cellular fractions, one also prevents obvious drastic changes in the samp before it is injected into another cell. Since the cellular material does r stick to the walls of the pipette, all material removed and measured with t volumetric submicromanipulator can be injected into another cell.

As previously described by Kopac,[9] both donor and receptor cells to be us in subcellular transplantation are first transferred to one of the fluorocarb fluids, of which Kel-F-I is perhaps the most satisfactory owing to its relativ low evaporation rate. Measured volumes of subcellular material are dra into a siliconized micropipette, and the sample is sealed within the micropipe at both ends by fluorocarbon seals consisting of small volumes of the same flu in which the cells are suspended. These seals protect the sample fr chance contamination and injury with aqueous media. The outer seal that injected with the sample serves as a marker and thereby indicates that t material was actually injected into the cell.

Another useful feature, involving fluorocarbon fluids, is that the cells followi micrurgy can be quickly converted into single-cell cultures[9] by surrounding t cell with the proper culture medium. Measured volumes of culture mediu ranging from 0.0005 µl. and upwards, can be conveniently added with a mic pipette. After the cell has "conditioned" the medium and has establishe subline, the culture may be transferred to more conventional tissue cultu preparations. Before this is done, new media can be added to replace the o by a microperfusion technique performed with micropipettes and suita volumetric controls.[8, 9]

Another advance is the television-micromanipulator (FIGURE 4). This strument, equipped with a monochromator and quartz or reflecting optics, m be used with either visible or ultraviolet light.[8] The present instrument cludes four Leitz micromanipulators and a semiquantitative volumetric cont unit. These will be modified later to accommodate one or more volumet submicropipette units.[19]

For this work, the conventional microscope stand is used. The mo chamber, integral with the mechanical stage, is sufficiently shallow to perm use of condensing systems with relatively short working distances. Even w hanging-drop preparations, the fluorocarbon technique eliminates the us difficulties experienced with cells floating in aqueous media whose densities near that of the cells. Since the fluorocarbons have a much higher density, t cells are spontaneously floated to the lower surface of the coverglass, wh they remain in place.[9]

Color-producing cytochemical reactions can be studied with essentia monochromatic light, since the Vidicon functions as a scanning-type light sorption indicator. Some of these reactions may be converted into semiquan tative estimates by interconnecting a selected, single raster line from t Vidicon with an oscillograph. This selected pulse appears on the oscillograp screen as a density curve while, simultaneously, the same pulse appears a white line on the Video screen.

One of the important applications of this new instrument, in reference transplantation experiments on nucleoli, is to observe the effects of such p

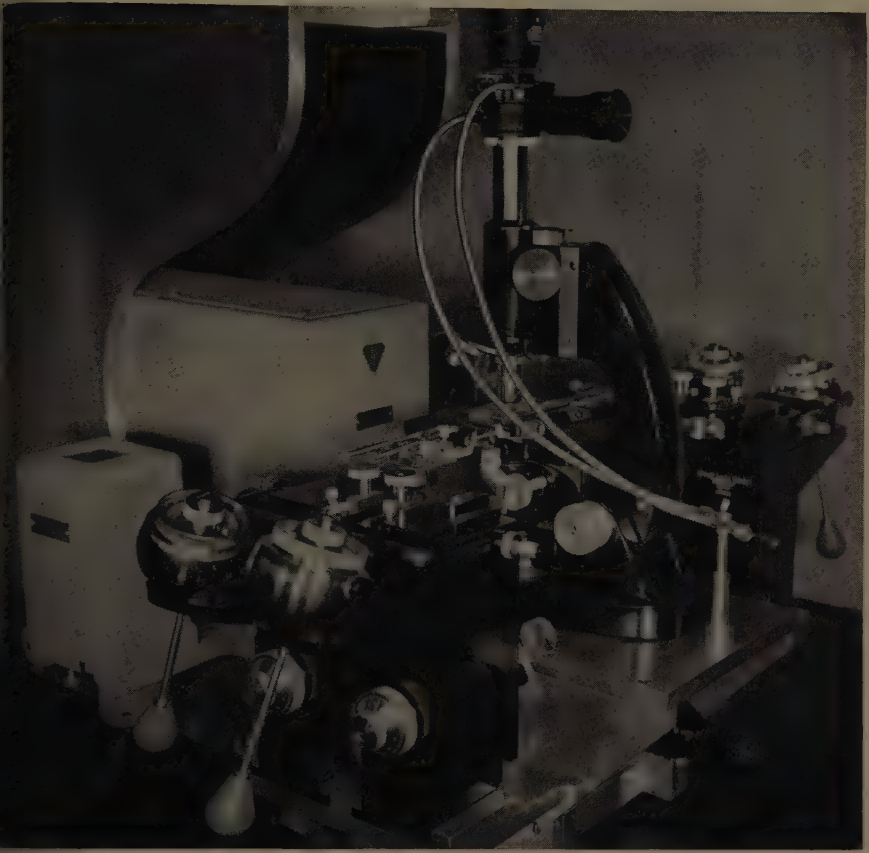

FIGURE 4. The television-micromanipulator. In the photograph are shown the following components:
nonocular microscope with Leitz Mikas attachment, Bausch and Lomb monochromator, with visible light source
ınd mounted in front of microscope, and a pair of Leitz micromanipulators mounted on either side. Not shown is
he RCA-Vidicon camera, mounted on the stand above the microscope and coupled to the Mikas attachment with
bellows. The video control unit and screen (partly visible behind monochromator) is placed in front of the
ight-hand pair of micromanipulators. Photographs are taken of the image on the video screen with a Polaroid-
and camera, which may be seen behind the objectives.

Individual raster lines, isolated with a line selector, and interconnected with an oscillograph permit densi-
ometric analyses of each line. A single nucleolus from the normal frog kidney cell extends over 10 to 20 raster
ines (initial optical magnification is about ×250).

All components are mounted as a single, integrated instrument on a massive aluminum alloy stand constructed
y Jack Harris. Below the subbase for the microscope may be seen a second lamp, used for heterochromatic
isible light. A first surface mirror, mounted on a horizontal slide, serves to focus light from the monochromator
o the objective through a substage objective that is used as a condenser.

The new Leitz micromanipulators each consist of lever-activated horizontal movements (ball-bearing slides)
nd a vertical motion activated by co-axial coarse and fine controls. In this quadruple micromanipulator, four to
s many as eight microneedles or micropipettes can be precisely controlled. Another feature is that the mechan-
cal advantage of the lever principle can be adjusted to meet the requirements demanded by the magnifications
mployed in viewing the microoperation.

edures on the stainability with, or the retention of, either methylene violet
Bernthsen)[21] or other thiazine dyes.[22]

The methods of microdilatometry have also been developed for estimating
he ribonuclease and phosphatase activities of small amounts of cellular ma-
erial.[19] These methods, in some instances, are sufficiently sensitive to esti-
nate the RN-ase activity of a single, large nucleolus. The principles of differ-

entiating between the enzymatic properties of cytoplasmic matrix and oth subcellular particulates have also been developed.[8]

Summation of Properties of Nucleoli

The notation of symbolic logic can be profitably applied towards the analys of the properties of nucleoli.[23, 24, 25] This permits one to set up the importa factors and their more probable relationships. In FIGURE 5, the properties of nucleolus are stated as a proposition. Eventually, as more information becom available, such propositions can be changed into linear equations. In view the experimental approaches to this problem, it is important to recognize t normal site of the nucleolus and also other sites that may be established b micrurgical methods. Symbolic notation is conveniently adapted towar this end as illustrated in FIGURE 6.

Transposition and Transplantation of Nucleoli

The basic plans in transposition and transplantation experiments are illu trated in FIGURE 7. Along with the diagrams are shown the symbolic not tions which permit one to state precisely where the nucleolus was and also whe it is placed. Attention is also given to the history of the cell serving as t donor of the nucleolus. For example, one may transfer a single nucleolus, part of a nucleolus, or one of several that may be present in the donor ce Similarly, one may insert the nucleolus into the cytoplasm of another cell th may or may not have its original nucleolus or nucleoli.

Obviously, the transposition experiments serve as a control for transplant tion experiments. This is probably the only means of controlling or evaluati the effects of genetic constitution on either the nucleolus or on the action of t nucleolus on its new environment.

At the present time, certain properties can be determined either directly indirectly. The effects of transposition or transplantation, summarized in symbolic proposition in FIGURE 8, may then be evaluated in terms of specif changes in any one enzyme or chemical constituent, in addition to changes in siz

$$\sum_{N}^{C}(n) \equiv \left[\begin{matrix}\left(\sum \exists e + E + \exists p + P\sum F\right)_n \\ \updownarrow \\ \left(\sum E + P\sum F\right)_{NC}\end{matrix}\right] (MV^{-1})_n \left[n \in N \subseteq C\right] t^{\alpha} \wedge \left[\cap T_n \wedge \cap G\right] t$$

FIGURE 5. Properties of nucleoli stated in symbolic notation. As stated symbolically, the sum of all pr erties of the nucleolus, between the limits of nucleus and cytoplasm, are logically related, ≡, to a series of facto The symbol ∃ is an existential modifier. Accordingly, the top line in the first bracket reads as follows: su Σ, of any one enzyme, $\exists e$, plus all other enzymes, E, plus any one chemical constituent, $\exists p$, plus all other chemi constituents, P, times the sum of free energy, F, in the nucleolus. The symbols, $\exists e$ and $\exists p$, represent any o enzyme or chemical constituent that can be experimentally determined. The lower line states: sum of all zymes, E, plus all chemical constituents, P, times the sum of free energy, F, in both nucleus, N, and cytoplas C. The arrow indicates the possible interrelation between these quantities. The free energy in this instance m consist almost entirely of energy-rich phosphates plus the mechanisms for generating energy-rich phosph bonds.

MV^{-1} represents the mass and volume (density) of the nucleolus. The second bracket states exactly where nucleolus is in reference to the nucleus and cytoplasm and also the relationship between the nucleus and cy plasm (see FIGURE 6). The symbol, ∧, represents the intersection of the quantities in the second bracket w those in the third bracket, *i. e.*, those quantities which have something in common. In the third bracket is in cated the intersection set, $\cap T_n$, of all structural aspects of the nucleolus which intersect or meet, ∧, with intersection set, $\cap G$, of the genetic factors. The implication here is that not all structural or genetic factors necessarily involved in determining nucleolar properties. Time is represented by t, while alpha and beta constants. Alpha and beta may or may not be the same, thus implying that time may not have the same eff on the location of the nucleolus as it may have in connection with structural or genetic factors.

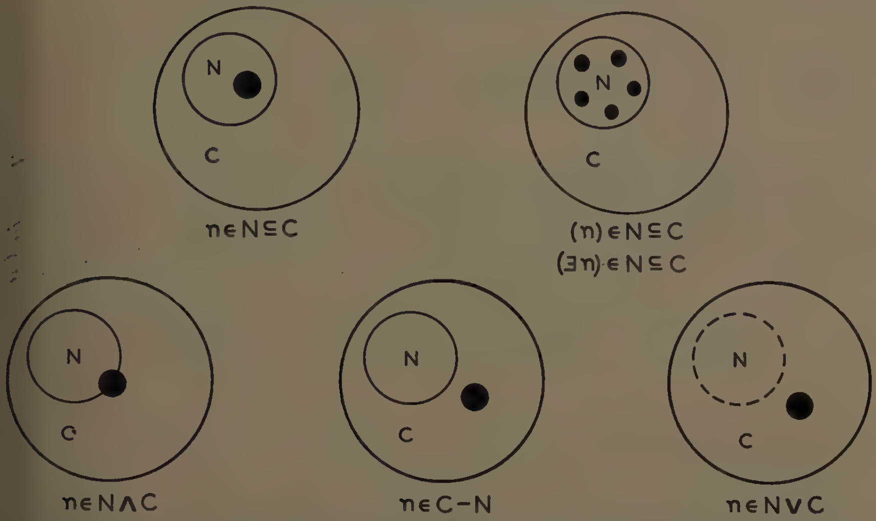

FIGURE 6. Diagrams of nucleolar positions with symbolic notation. In the upper left, the proposition is that the nucleolus (black circle), *n*, is an element, ϵ, of the nucleus, *N*, which is a subset, $\subseteq$, of the cytoplasm, *C*. In the upper right, the proposition is that all nucleoli, (*n*) are elements of the nucleus which is a subset of the cytoplasm, or that any one nucleolus, ($\exists$n), is an element of the nucleus. In the lower left, the nucleolus is an element of both, $\wedge$, the nucleus, *N*, and the cytoplasm, *C*, as would be produced if the nucleolus becomes partly extruded from the nucleus. In the lower center. the nucleolus is an element of the cytoplasm, *C*, but not of the nucleus, *N*. This situation would occur following complete extrusion, or by transplantation as produced micrurgically. The lower right diagram states that the nucleolus is en element of either, $\vee$, the nucleus or the cytoplasm, or both. In this instance, the cell is undergoing mitosis and the nucleolus was introduced by micrurgy.

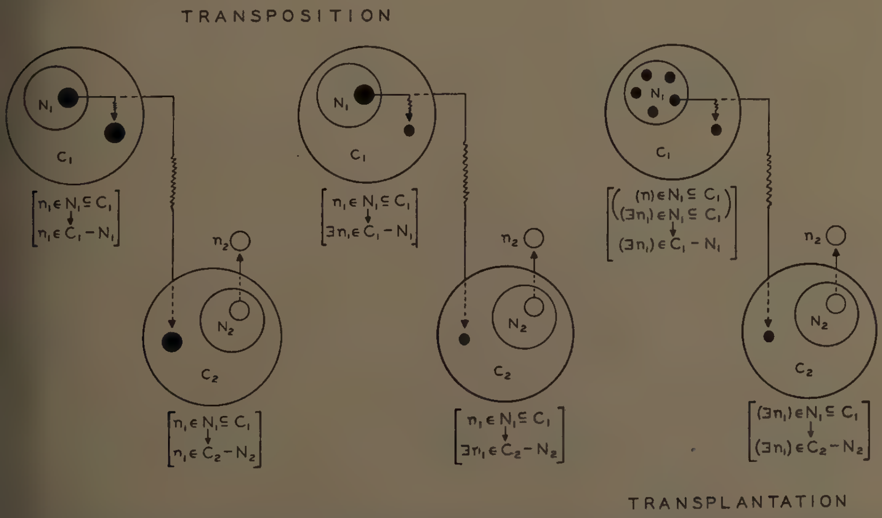

FIGURE 7. Transposition and transplantation of nucleoli. There are two propositions in each bracket, the upper one states where the nucleolus was and the lower one indicates where the nucleolus was transferred, by micrurgical methods. The subscripts, indicate the identity of the cell. If the subscripts are the same, the operation is one of transposition. if different, the operation is one of transplantation. In this diagram, three possibilities of transposition or transplantation are shown, *i. e.*: entire nucleolus, *n*, part of a nucleolus, $\exists n$, or at least one of several nucleoli, ($\exists n$).

All transposition and transplantation experiments included here indicate the removal of a nucleolus, n_1, from the nucleus and implanting it into the cytoplasm of the same, 1, or different cell, 2. In transplantation experiments, the nucleolus, n_2, originally possessed by the host cell may or may not be removed prior to the transplantation of nucleolus, n_1.

$$\left[\begin{matrix}\exists e\\ \overline{\exists p}\\ \overline{MV^{-1}}\end{matrix}\right]_n^{-1} \equiv \overset{*}{\left[\begin{matrix} n_1 \in N_1 \subseteq C_1 \\ \downarrow \\ n_1 \in C_1 - N_1\end{matrix}\right]} t^{-\alpha} \wedge \left[\bigcap_1^2 T_n \wedge \bigcap_1^2 G\right] t^{-\beta} \left[\begin{matrix}(\sum E + P\sum F)_n \\ \updownarrow \\ (\sum E + P \sum F)_{NC}\end{matrix}\right] \left[\sum_N^C (n)\right]$$

FIGURE 8. Evaluation of effects following transposition or transplantation, stated symbolically. T proposition states that any one enzyme, $\exists e$, or any one chemical constituent, $\exists p$, or mass, volume, or densit which may be experimentally determined, are logically related, $\equiv$, to the terms that follow. In the first brack (marked with asterisk) is indicated the type of operation produced (see FIGURE 9) and is based on the notati described in FIGURES 6 and 7. The change in nucleolar position then intersects, Λ, with quantities in the seco bracket. Here, attention is focused on the possible effects of changing the nucleolus from one part of a cell another or from cell 1 to cell 2, on both intersection sets of structural aspects, $\bigcap_1^2 T_n$, and genetic factors, The role of time is also symbolically noted. The third bracket includes the intersection of enzymes, chemi constituents, and free energy of the nucleolus with those of either the cytoplasm or nucleus, as indicated in FI URE 5. In the fourth bracket, the summation of all properties of the nucleus between the limits of nucleus a cytoplasm is indicated.

shape, or other structural features. Such effects following the transfer, mu always be considered in two ways: (1) effects on the nucleolus, and (2) effec on the site.

As more data become available so that the symbolic propositions, as show in FIGURE 8, can be converted into linear equations, the effects of genetic co stitution and structural features on the nucleoli may then be evaluated. Suc linear equations obtained for each operation involving the nucleolus can the be combined as a system into matrices and, following this, matrix theory ar algebra can be applied.[26]

Several examples of transposition or transplantation are diagrammed wi symbolic notations in FIGURE 9. For each experiment, one indicates whe

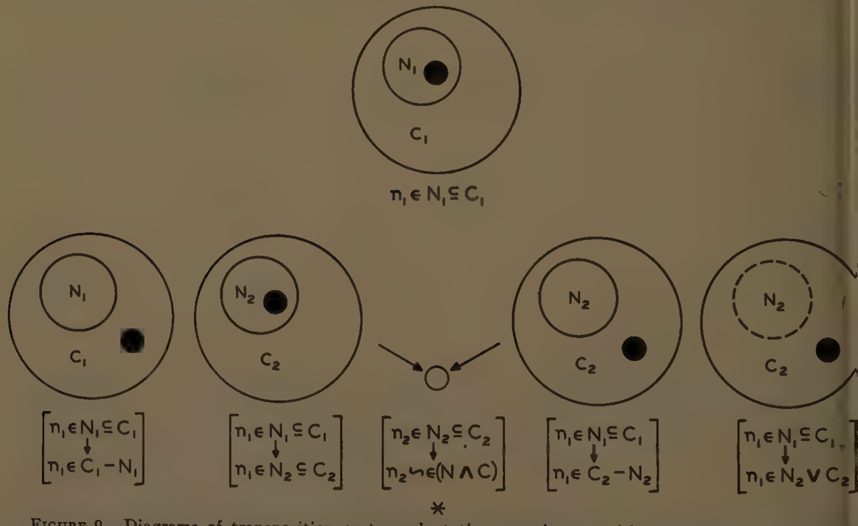
N₁
C₁
n₁ ∈ N₁ ⊆ C₁
N₁
C₁
N₂
C₂
N₂
C₂
N₂
C₂
n₁ ∈ N₁ ⊆ C₁ ↓ n₁ ∈ C₁ − N₁
n₁ ∈ N₁ ⊆ C₁ ↓ n₁ ∈ N₂ ⊆ C₂
n₂ ∈ N₂ ⊆ C₂ ↓ n₂ ∽∈ (N ∧ C)
*
n₁ ∈ N₁ ⊆ C₁ ↓ n₁ ∈ C₂ − N₂
n₁ ∈ N₁ ⊆ C₁ ↓ n₁ ∈ N₂ V C₂

FIGURE 9. Diagrams of transposition or transplantation experiments with symbolic notation. In ea bracket, the upper line indicates where the nucleolus was, and the lower line states where the nucleolus w placed, following micrurgy. The experiment is transposition if the subscripts are the same, while the experime is designated as transplantation if the subscripts are different.

Thus, in the second figure from the left, a nucleolus, n_1, was removed from cell 1 and placed into the nucle of cell 2, which may or may not have its original nucleolus, n_2. In the lower right diagram, the nucleolus fr cell 1 was transplanted into the cytoplasm of cell 2, which is undergoing mitosis. In the center is indicate nucleolus removed from cell 2 and placed into the surrounding medium. The lower line in the bracket states th the nucleolus, n_2, is not, $\sim$, an element of either the nucleus or cytoplasm of any cell.

the nucleolus was, its history in reference to other nucleoli in the cell, and also where such nucleoli were placed following micrurgy.

Specific attention should be drawn to the opportunity, with these experiments, of learning something about the relation of gross morphology to nucleolar properties. In neoplastic cells, as reported by Long and Taylor,[27] it is not unusual to find multiple nucleoli. Also, one finds nucleoli of unusual size or shape.[28] Frequently, these nucleoli have rather large, single, or multiple, vacuoles. Fragmentation is apparent in some; consolidation in others. All these structural aspects are, at the present time, combined as the intersection set, $\cap T_n$, pertaining to nucleolar structure (FIGURES 5 and 8).

Evaluation of Nucleolar Changes Following Transposition or Transplantation

One of the more useful first approximations involves the rather specific supravital staining of the nucleolus by methylene violet (Bernthsen).[21] Not only can one observe the effects of transposition or transplantation on the retention of methylene violet, but one can also determine the effects of experimental treatment on the subsequent stainability of nucleoli. In other words, will the nucleoli accumulate methylene violet as readily if transferred to cytoplasm as they do in their normal nuclear environment?

Changes in dye retention or stainability can be readily detected and measured, especially with the television-micromanipulator-oscillograph. These determinations will therefore serve as a means of obtaining the first indications of nucleolar changes following transplantation. Obvious differences in reactivity with methylene violet would indicate a change in the properties of nucleoli. On the other hand, if no changes are evident with methylene violet, still other changes might have occurred. In such instances, the more highly refined and complex procedures would be required. The following summary gives a list of various properties of nucleoli that can be evaluated:

RNA and DNA content	Ultraviolet light absorption
	Staining with basic and acidic dyes
	Supravital staining
Lipid content	Staining, extraction, *etc.*
RN-ase activity	Microdilatometry
Phosphatase activity	Microdilatometry
Action of RN-ase	Cytochemical micrurgy
Density	Sedimentation rates
Mass	Interference microscopy
	Soft X-ray absorption
Volume	Direct measurement and calculation
	Calculation from mass and density data
Gross morphology	Microscopic observation and comparison
Fine structure	Electron microscopy

Nucleolar Interactions with Other Cellular Structures

In either transposition or transplantation experiments, the cytoplasm of the ame or different cells serves as a physicochemical substrate permitting certain eactions, albeit of considerable complexity and subtlety. Some of the anticpated interrelationships are summarized in FIGURE 10. Especially highighted is the interplay of enzymes of the nucleus and cytoplasm with the

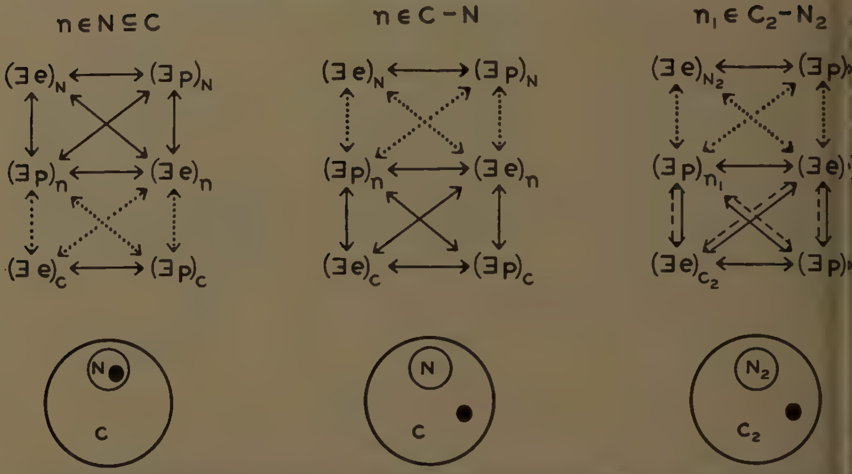

FIGURE 10. Diagrams showing relation of nucleolus to nucleus and cytoplasm. In the left diagram is i cated the interaction of any one enzyme, ∃*e*, with any one chemical constituent, ∃*p*, of the nucleolus, *n*, with th of the nucleus, *N*, or cytoplasm, *C*. The solid lines with arrows indicate the most probable relationships, w the dotted lines indicate possible, although improbable, relationships due to the potential barrier effects of nuclear membrane. This would be normal for a nucleolus, *in situ*.

Transfer of the nucleolus to the cytoplasm may be expected to establish different relationships, as indica in the center diagram, with cytoplasmic constituents, since the potential barrier effect of the nuclear membr is no longer a factor in reference to ∃*e* and ∃*p* of the cytoplasm. Interactions with similar constituents in nucleus are now less likely.

The expected relationships following transplantation of nucleolus from the nucleus of cell 1 to the cytoplas cell 2 are shown in the right diagram. The resulting effects may be the same (solid lines) as those obtained foll ing transposition (center). On the other hand, with possibly different genetic backgrounds and other fac (see FIGURE 8), there may be different interactions, as indicated by the broken lines.

chemical constituents and enzymes of the nucleolus. The transfer of the nucle lus to the cytoplasm may be expected to create new relationships owing to t possible barrier effect of the nuclear membrane to the exchange of certain e zymes and constituents. Moreover, it is to be expected that some of the e zymes and chemical constituents of the cytoplasm will differ from those prese in the normal nucleolar environment.

The effects of genetic differences should show up on comparing nucleo behavior following transposition and transplantation. Assuming that the r cleolus was obtained from cell 1, its transfer to the cytoplasm of cell 1 wou establish the effects of a cytoplasmic environment (of identical genetic co stitution) as compared with the original effects induced by the environme provided by the nucleus. The transfer of a nucleolus from cell 1 to the cyt plasm of cell 2 may or may not produce the same changes. If the effects the nucleolus are not the same, then one may assume differences between t genetic backgrounds of cells 1 and 2. On the other hand, if there are no sign icant differences, then either the genetic constitutions of the two cytoplasm must be essentially alike, or else the genetic backgrounds are incapable modifying the transplanted nucleolus.

Of special significance, afforded by transplantation experiments, is the c portunity of determining the effects produced on nucleoli by a cellular syste during mitosis. At this time, the nucleoli of cells in karyokinesis do not ha their traditional gross, compact structure, but their nucleolonemata now a pear to be arranged in an entirely new structural pattern and are apparently closely associated with certain chromosomes (Estable and Sotelo[29]). To this system may now be added a "compact" nucleolus obtained from another cell during interkinesis. Experiments of this type can be evaluated only if the prior effects of different cytoplasms on nucleoli are known (following transplantation into interkinetic cytoplasm).

Also to be determined are the effects of extraneous nucleoli on karyokinetic and subsequent cytokinetic processes. Another point that can be clarified is whether a cell needs a nucleolus in order to initiate and complete karyokinesis and cytokinesis. For such experiments, the interkinetic nucleolus is removed with a micropipette.

Along with changes that may be induced in nucleoli, following transposition or transplantation, other important effects can be studied that are focused mainly on what the nucleolus does to the cell. Some of such effects may be noted in the cytoplasm in terms of changes in nucleic acids, structure of particulates, *etc.* On the other hand, these changes may be much more subtle and can perhaps be indicated only by maintaining such cells at least long enough for them to reproduce, if possible. Furthermore, it may be necessary to apply not only classical cytologic methods but also electron microscopy and cytochemical methods that are yet to be developed.

It is clear that to establish the properties of nucleoli as functions of their interactions with cytoplasm, with other nuclei, with other cytoplasms, or with cells in karyokinesis, it is essential to employ quick and simple methods for checking possible changes in nucleoli. For this reason, the interaction of nucleoli with methylene violet or other supravital dyes followed by television-oscillographic analyses should be the most practical.

Extension to Other Subcellular Components

Although emphasis has been placed on nucleoli in this report, the same experimental and theoretical procedures are directly applicable to a similar attack on several problems involving the properties and functions of other subcellular particulates. Of these, a few are singled out for special consideration.

Chromosomal transplantation. Chromosomes become clearly defined and frequently accessible for isolation and transplantation during mitosis. With careful and precise micrurgy, chromosomes can be removed and transplanted to other cells. In this way, aneuploidy is synthetically produced. Especially useful for such studies would be cells obtained from neoplasms of the Chinese hamster (*Cricetulus griseus*) since these rodents have only 11 pairs of large and morphologically distinguishable chromosomes (Yerganian[30]).

Production of polyploidy. The frequent occurrence of polyploidy in various types of ascites tumors in mice (Hauschka and Levan[31]) and other animals provides excellent material for exploring the probable causes of polyploidy by transplantation experiments. One series of experiments could settle the question whether the cytoplasm from cells that are consistently tetraploid will induce tetraploidy if injected into normally diploid cells. In a similar way, the cytoplasm of octaploid cells could be tested on normally diploid or tetraploid

cells. Another question to be answered is whether the cytoplasm of diplo
cells will inhibit the polyploid condition.

At present, there is, no general information about the endogenous origin
polyploidy except as suggested by the observations of Hsu and Moorhead
Transplantation experiments may settle the issues as far as the probable r
of cytoplasm is concerned. Assuming that cytoplasm does play a role in elic
ing polyploidy, what volume of this particular cytoplasm would be required
elicit or inhibit the effect? For such studies, volumetric transplantation
comes essential. In addition to the feasibility of establishing single-cell cu
tures, with the fluorocarbon technique, there is the further possibility of
tablishing clones by inoculating newborn mice with the experimental ce
(Hauschka[33]).

Transplantation of cytoplasm from melanocytes to amelanocytes. The melan
cytes and amelanocytes from piscine and other sources can be cultured
fluid media. Such material permits a variety of interesting transplantati
experiments, including the transplantation of cytoplasm from the melanocy
to an amelanocyte.[34] By the use of the volumetric submicromanipulator a
the fluorocarbon technique, measured volumes of cytoplasmic substance can
transferred to cells that subsequently may be converted into single-cell cultur
The first point to establish is whether an amelanocyte can be converted int
melanocyte by seeding it with a small volume of melanocyte cytoplasm.
such a conversion occurs, is this due to the multiplication of melanocyte pigme
granules or to the conversion of amelanocyte granules? Will melanocytes
come amelanotic if their cytoplasm is seeded with granules from amelanocyte
Will amelanotic granules become melanotic in the cytoplasm of melanocyte

Numerous cytochemical tests can be employed to test the action of tra
planted cytoplasm on the host cell as well as to determine the effect of h
cytoplasm on the transplanted sample. Dopaoxidase activities can be c
veniently demonstrated, while silver methods (Masson[35]) will help identify t
pigment granules. The uptake of radiotyrosine can also be indicated by n
clear track autoradiographs, using NTB$_3$ emulsions.[36, 37]

Inclusion bodies in frog renal adenocarcinoma. The intranuclear and cyt
plasmic inclusions frequently associated with the renal adenocarcinoma of t
frog[38, 39] can also be investigated by transposition and transplantation coupl
with cytochemical micrurgy. Duryee[40] was one of the first to recognize t
potential value of subcellular transplantation in cells of this tumor.

The important problem to determine is to what extent these acidophilic
clusions are responsible for the conversion of normal kidney cells into ade
carcinoma cells. Are the effects following transplantation of the inclusi
bodies dependent on the form assumed by the bodies prior to transplantatio
In other words, would the same action be induced in a host cell following tra
plantation, if the inclusion bodies were compact, diffuse, or extruded into t
cytoplasm?

Does the nuclear inclusion become infective if it remains in the nucleus
must it be extruded so that its effects are produced only under extranucle
conditions? Must the inclusion body interact with the cytoplasm, followi

ts extrusion,[39, 40] before it can become an infective agent? Can the same
ffect be produced by transferring the inclusion from the nucleus to the cyto-
lasm with a micropipette (transposition)?

From such studies, the relationship of the inclusion bodies to the neoplasm
hould be established. The mechanism of infectivity of cells, if such a process
ccurs, can also be elucidated at least as far as the conditions during which the
apacity of infectivity is developed. Whenever certain changes in morphology
f cells accompany the malignant condition, *i. e.*, enlarged or multiple nucleoli,
olyploidy, hyperchromatism, cellular, or intranuclear inclusions, there is always
he question whether these changes are the cause or the result of malignancy.
he application of subcellular transplantation to the inclusions found in the
ucké adenocarcinoma may, in time, yield enough information so that one can
etermine whether these inclusions are the inciters or the effect of malignancy
n this neoplasm.

Summary

This report summarizes certain theoretical and experimental aspects of sub-
ellular transplantion utilizing symbolic notation, topology, structural, and
enetic factors, cytochemical micrurgy, fluorocarbons, single-cell cultures, vol-
metric submicromanipulation, and the television-micromanipulator. High-
ighted in this report are studies designed to give information concerning the
roperties and function of nucleoli. The extension of these procedures to the
ransplantation of various cytoplasmic particulates, chromosomes, intranuclear,
r cytoplasmic inclusion bodies associated with the kidney adenocarcinoma of
he frog, is discussed. The results of these investigations wil be reserved for
uture publications.

It is reasonable to expect that any success in transplantation experiments
ill depend on the compatibility of the donor subcellular fraction with the host
ytoplasm. This would imply that the donor-host cells should be similar, thus
ecognizing the role of genetic constitution. Accordingly, one should expect
ore favorable compatibility in melanocytes and amelanocytes from the same
umor, in kidney cells and kidney carcinoma cells obtained from the same frog,
amebas from the same clone, or in ascites tumor cells from single-cell sublines.

The transplantation procedures must be so well rehearsed that only the
hortest time should elapse between removal of the sample from one cell and
s injection into another cell. In addition to the development of essential
kills, the successful transfer of subcellular particulates requires superb micrurgi-
al instrumentation and techniques. Some of these have already been ad-
anced, including, for example, the volumetric submicromanipulator, the
levision-micromanipulator, as well as the fluorocarbon techniques.

Other instrumentation and techniques may need to be developed to permit
reater approaches to perfection. No instrument or method has ever been
esigned that cannot be improved. As an example, Harris and Kopac[41] have
ecently developed an entirely new microscope-micromanipulator, in which the
icroscope is modified specifically for micrurgical purposes and is integrated
ith four micromanipulators of improved design. Instruments such as these

will obviously insure greater success in executing critical experiments in wh subcellular fractions are transplanted.

Acknowledgments

These investigations were suported in part by a research grant (C-2018) fr the National Cancer Institute, of the National Institutes of Health, Uni States Public Health Service, Bethesda, Md., and by grant DRG-271 from Damon Runyon Memorial Fund for Cancer Research, Inc., New York, N.

The volumetric submicromanipulator, the microdilatometer, and the te vision-micromanipulator were constructed by Jack Harris, whose skills as instrument designer and maker are gratefully acknowledged.

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Part IV. The Cytology of Early Cancer

DYSPLASIA OF THE UTERINE CERVIX

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The term dysplasia can be used to designate a group of heteroplastic lesi
involving the surface epithelium of the uterine cervix that are basically cha
terized by a relative or an absolute increase in the number of immature c
and by certain manifestations of abnormal differentiation.

Similar or related changes have been described in the medical literature
der a varied terminology including atypia,[12, 1, 10] atypical,[8, 9] or irreg
epithelium,[4, 19] anaplasia,[20] precancerous metaplasia,[2] dissociated intraepithe
anaplasia,[17] basal cell hyperplasia[5, 7, 14] or hyperactivity,[5, 6] atypical hyp
plasia,[13] dyskaryosis,[15] leukohyperkeratosis,[18] and leukoparakeratosis.[18]
literature also includes numerous lesions classified as carcinoma *in situ* wh
histopathological changes are similar to those under consideration.

The dysplastic lesions vary not only in regard to their content of abnorm
or primitive cells, but also as to the relative degree of immaturity of th
elements. This, in part, explains the multiplicity of terms that have b
applied to these changes. When the magnitude of the dysplasia is great,
changes approach those observed in carcinoma *in situ* and warrant a m
serious consideration. On the basis of our present knowledge, a slight deg
of dysplasia is relatively unimportant, being more analogous to lesions ca
gorized as "metaplasia with atypicality"[11] or even so-called "basal cell hyp
plasia."[5, 7, 14]

This study is based on 100 cases of histopathologically proved dyspla
Cellular preparations were obtained by scraping the uterine cervix and aspir
ing the contents of the cervical canal. Using a systematic meander scann
pattern designed to cover the entire slide, the features and measurements
altered cells were recorded at regular intervals in an attempt to provid
random sample of the cellular specimen. The entire slide was studied in or
to count the number of altered cells present and to record and identify
groupings of the cellular elements. Similar studies were made on 100 case
in situ cancer and 100 cases of invasive cancer, all of which were verified
biopsy.

Histopathology

The critical area in dysplasia lies in proximity to the external cervical
The change is usually observed in the mucosa in relation to the external cerv
os, although it may extend to involve the epithelium covering the po
vaginalis.

On histopathological examination, the dysplastic epitheliumlike nor
stratified squamous epithelium may have a rather distinct stratum cylindric
or basal cell layer. The overlying zone analogous to the deep spinous laye

ormal stratified squamous epithelium is ill-defined in dysplasia and contains greater number of primitive cells, as do the more superficial layers of the pithelium. Thus, as the surface is approached, there is less evidence of ellular maturation in dysplasia as compared to normal stratified squamous pithelium. The greater immaturity of the component cells in dysplasia is videnced by their smaller size, their relatively large nuclear masses, cytolasmic basophilia, alterations in the cytoplasmic fibrillar apparatus, and abent or diminished glycogen content (FIGURES 1 and 2).

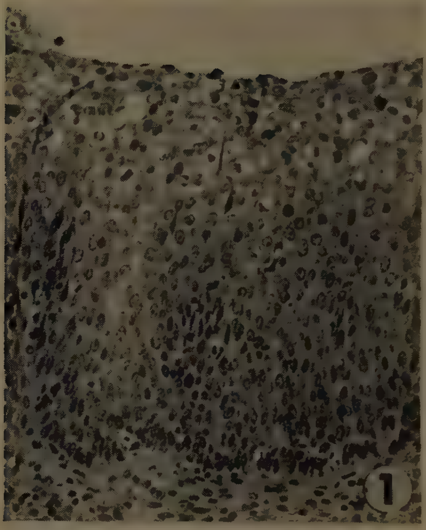
There is active evidence of cellular proliferation in some of the dysplasias. hese have an increased number of mitotic figures that may be either normal r abnormal. Changes in the structure and the staining reaction of the nuclear hromatin are also associated with active proliferation. Alteration in the olarity of the cells is probably the direct result of rapid or abnormal cellular roliferation. Other lesions are not actively proliferating when examined, but re characterized by changes suggesting a previous period of cellular multipliation. Some of these are associated with evidence of regressive changes FIGURE 4).

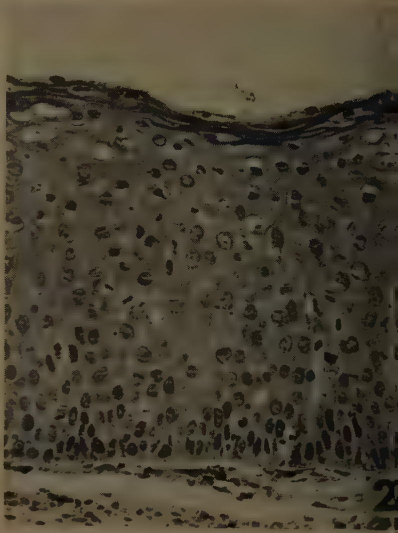
Certain abnormalities of cellular differentiation characterize many of the ysplastic lesions. This is manifested either as isolated cellular keratinization hroughout the epithelium (FIGURE 4) or as a rather sharply demarcated change nvolving the superficial strata of the epithelium, overlying the intraepithelial yer. The latter may be an excessive formation of keratin (FIGURE 4) with n underlying granular layer that is distinctly abnormal for a mucosal surface. his may exist alone or in combination with premature keratinization, resultng in a change comparable to the parakeratosis sometimes seen in altered idermis (FIGURE 3).

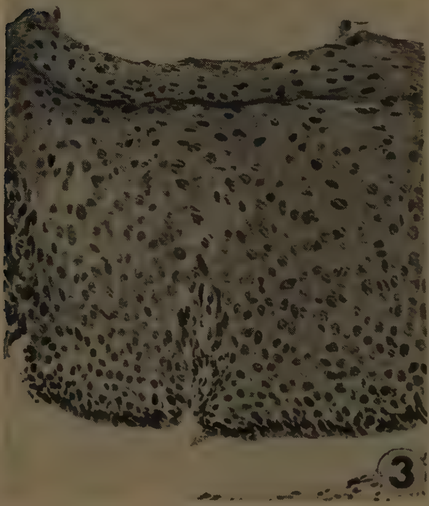
Cellular Changes

When the histopathological changes in dysplasia are correlated with cellular udies, it is apparent that alterations in the surface epithelium are reflected in e desquamated cells. As a result, considerable knowledge of the underlying athological process can be gained by studying the cellular specimens.

The number of altered cells observed in cytological preparations is of some nportance. While counts of abnormal cells vary from one case to another nd may fluctuate considerably in any given case, in general, fewer abnormal lls are removed from predominantly dysplastic lesions as compared to *in situ* ancer. This has been demonstrated in a previous study.[16] Similarly, in eneral, the number of altered cells observed in specimens obtained through e use of physical force is probably related to the degree of dedifferentiation the altered epithelium. Coman[3] has previously shown that cancer cells are ss adherent to one another as compared to normal cells. Thus, there is ason to believe that there is decreased mutual adhesiveness in the cells of *situ* cancer. Since the dysplastic cells are relatively more mature, they are obably more adherent to one another and are less readily separated. Other ctors, including the extent of the process in the surface epithelium may have me bearing on the number of abnormal cells observed. These, however, are obably of secondary importance.







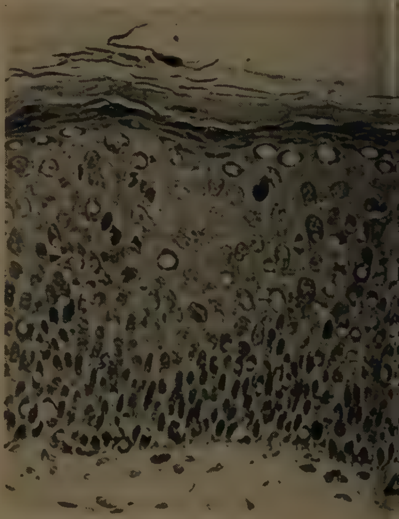


FIGURE 1. A marked degree of dysplasia. There is little evidence of maturation as the surface is approac however, the cell borders remaining distinct. This lesion was observed in a patient who, 10 months later, pr to have *in situ* cancer.

FIGURE 2. A moderate degree of dysplasia. Although there is little evidence of maturation as the sur is approached, the immature forms are less numerous as compared to FIGURE 1. This lesion has persisted f months during which time the patient had two pregnancies.

FIGURE 3. A moderate degree of dysplasia. Overlying the intraepithelial layer there is a surface ch somewhat analogous to the parakeratosis observed in epidermis. This lesion persisted for 15 months but not demonstrated in subsequent studies made over a period of 50 months.

FIGURE 4. A moderate degree of dysplasia. There is marked vacuolization of the component cells and lated keratinization of one cell. The surface shows hyperkeratosis with an underlying granular cell layer. C plete removal of the change precluded follow-up studies.

The size of the altered cells is similarly significant. As determined by a librated ocular micrometer, 2500 mature squamous cells derived from the rvix had a mean diameter of 45 microns, 5000 altered cells in dysplasia had mean diameter of 37 microns, while the mean was only 23 microns in 6500 lls arising in carcinoma *in situ*. From distribution curves, it is apparent at many of the dysplastic cells fell within the range of the normal cells. is may be attributed to the fact that many of the dysplastic cells are rela- ely mature and that their spacial dimensions are thus comparable to those the normal cells. The majority of the dysplastic cells were of moderate size, d some forms were relatively small. In carcinoma *in situ*, there is overlap- ng of the distribution curve with that noted in dysplasia. This is explained, part, by the fact that about 80 per cent of the *in situ* cancers have a coexist- g dysplastic focus. The altered cells from this site may be dislodged along th those of the *in situ* cancer. Similarly, specimens from some of the pre- minantly dysplastic lesions contain cells whose measurements are comparable those noted in carcinoma *in situ*.

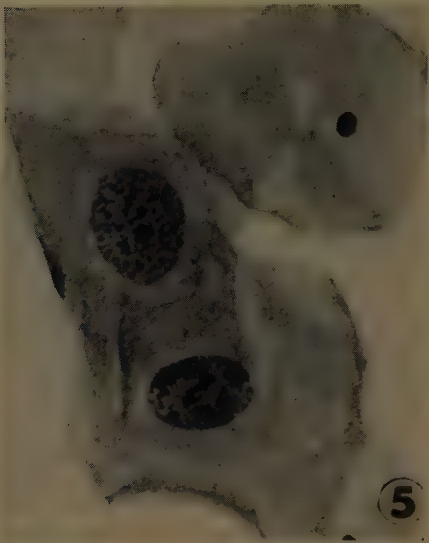
The configuration of the altered cells is also important. Selecting cells by systematic meander scanning pattern, configurations were recorded for 5000 tered cells in 100 cases of dysplasia, for 10,000 cells observed in 100 cases of *situ* cancer, and for a comparable number of cells from 100 cases of invasive ncer. Thus, TABLE 1 is based on a study of 25,000 cells.

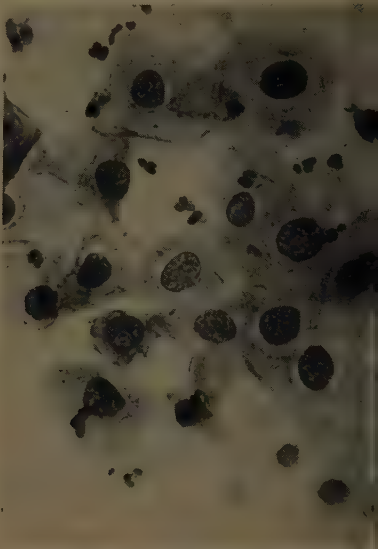
In dysplasia, 52.68 per cent of the cells were polyhedral in configuration as mpared with 8.19 per cent in carcinoma *in situ*, and only 2.05 per cent in vasive cancer. The round and oval forms accounted for about 41 per cent the cells in dysplasia, about 84 per cent in carcinoma *in situ*, and about 76 r cent in invasive cancer. The differences in the remaining categories are s striking. Ellipsoidal forms are slightly more common in dysplasia, while ngate, irregular, and so-called tadpole forms are somewhat less numerous. is evidence also indicates that the dysplastic lesions are more differentiated, d that the *in situ* and invasive cancers are more anaplastic.

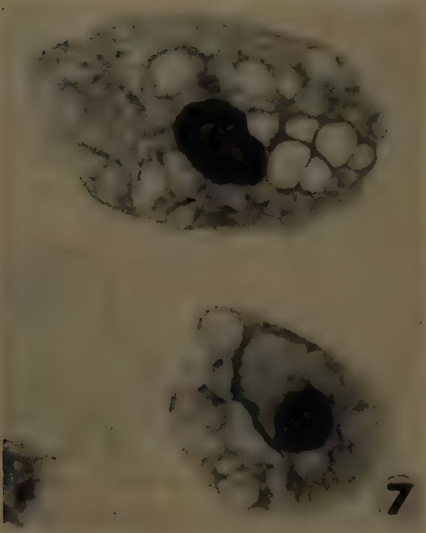
Similar evidence is gained by studying the arrangements of the cells. About per cent of the altered dysplastic cells are arranged in sheets. In such a ouping, the component cells are regularly arranged with relationship to one other and have distinct cell borders. About 0.2 per cent of the cells in splasia were arranged in syncytial masses. In such an aggregate, the com- nent cells are irregularly arranged with relationship to one another and have listinct cell borders. These are much more common in carcinoma *in situ* d invasive cancer, and are probably manifestations of altered polarity and oid cell growth.

Sheetlike arrangements were common, being noted in 98 of the 100 cases of splasia, while syncytial masses were noted in only 9 of the cases. These, thout exception, were associated with severe dysplasias.

In general, the size of the nuclear mass is an indication of the maturity of cell. As measured by the use of optical micrometer, the mean nuclear meter in 2500 mature cervical squamous cells was about 7 microns, while 5000 dysplastic cells the mean nuclear diameter was 14 microns and, in 6500







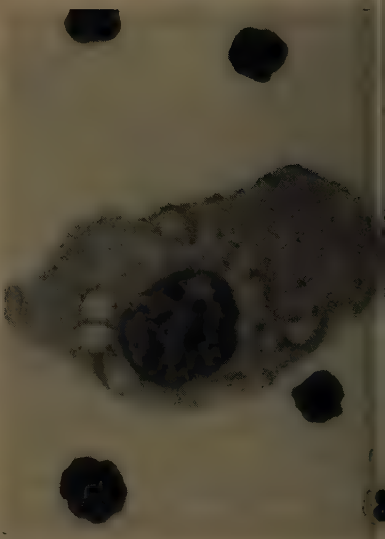


FIGURE 5. There are two large polyhedral cells from dysplasia with a normal mature squamous cell. dysplastic cells, the nuclear forms are enlarged and show an altered chromatin pattern and hyperchrom ×750.

FIGURE 6. A sheetlike arrangement of dysplastic cells. The alterations in the individual cells are s to those described in FIGURE 5. ×400.

FIGURE 7. Cellular elements from dysplasia in the human. There is marked cytoplasmic vacuolizat these cells which is not uncommon. ×1000.

FIGURE 8. An altered polygonal cell observed in vaginal fluid from a mouse with developing carcino the uterine cervix. Compared with FIGURE 7. ×1000.

TABLE 1

CELLULAR CONFIGURATION

	Dysplasia	*In situ* cancer	Invasive
lyhedral	2634 or 52.68%	819 or 8.19%	205 or 2.05%
al	1051 or 21.02%	3141 or 31.41%	3053 or 30.53%
und	1029 or 20.58%	5263 or 52.63%	4598 or 45.98%
ipsoidal	200 or 4.00%	167 or 1.67%	54 or 0.54%
ngate	75 or 1.50%	176 or 1.76%	1508 or 15.08%
egular	10 or 0.20%	408 or 4.08%	517 or 5.17%
dpole	1 or 0.02%	26 or 0.26%	65 or 0.65%

The configuration of altered cells as observed in a systematic meander screening pattern designed to er the entire slide. A total of 5000 cells were examined in dysplasia, and 10,000 cells were examined in inoma *in situ* and invasive cancer.

ls from *in situ* cancer, the mean was 13 microns. As compared with the rmal, the absolute nuclear diameter is larger in both dysplasia and *in situ* ncer, and the distribution curves are somewhat comparable. When the clear diameter is considered in relation to the cytoplasmic diameter, however, is apparent that the relative nuclear enlargement is greater in carcinoma *in* *u* as compared with dysplasia.

The nuclear mass stains more intensely in the altered cells and also shows rtain differences in basic structure. A predominantly finely granular chro- tin was observed in 94 per cent of the cells derived from dysplasia, while a rsely granular chromatin was more common in carcinoma *in situ* and in asive cancer. Translucent or opaque nuclear masses were more common in asive cancer (TABLE 2).

There were coexisting cells derived from superficial parakeratosis in 84 of 100 cases of dysplasia. These are relatively small, usually round, oval, or egular in outline. Their cytoplasm is either basophilic or acidophilic, and orange or yellow staining reaction is not uncommon. The nuclei are small d pyknotic.

Anucleate squamae derived from hyperkeratosis were observed in 44 of the cases of dysplasia. These, in many instances, were associated with cells taining cytoplasmic granules derived from a granular cell layer.

Occurrence

An estimation of the prevalence of dysplasia can be made by utilizing data tained through the use of a screening procedure. Among 10,553 cellular

TABLE 2

NUCLEAR CHROMATIN

	Dysplasia	*In situ* cancer	Invasive
ely granular	4720 or 94.40%	2198 or 21.98%	3418 or 34.18%
rsely granular	200 or 4.00%	7157 or 71.57%	4398 or 43.98%
que	80 or 1.60%	645 or 6.45%	2184 or 21.84%

The chromatin patterns in altered cells as observed in a systematic meander scanning pattern designed over the entire slide.

examinations submitted from physicians' offices and large survey cent there were 81 in which the cellular changes suggested dysplasia. This re sents an over-all prevalence of 0.77 per cent, which is comparable to the v of 0.6 per cent reported by Lapid and Goldberger.[12] Using a similar approa the prevalence of dysplasia in 1000 pregnant clinic patients who were dominantly Negro was about 3 per cent, although approximately 6 per cen the women displayed some degree of cellular abnormality. In general, plasia is more than twice as common in the Negro as in the white wom Carcinoma *in situ*, similarly, is more common in the Negro, although the o all prevalence in our experience is only about 0.39 per cent.[17]

Age at Detection

The mean age at detection in 200 cases of dysplasia is about 34 years w in 170 cases of *in situ* cancer proved by histopathological studies in this stitution, the average age was 41.5 years. Thus, dysplasia is detected at earlier age than *in situ* cancer.

Significance

In order to learn the significance of the change, over 200 women with plasia are now being followed in a special clinic at the University Hospital Cleveland, Ohio. Although it is known that dysplasia can be demonstrate about 80 per cent of the cases of *in situ* cancer and may also coexist with vasive cancer, any definite conclusions as to the relationship of dysplasia cancer based on follow-up studies involves a protracted period of surveillan since the latent period involved may be in excess of 10 years.

As of the present, about 60 per cent of the lesions apparent on initial bio are not demonstrated in subsequent studies. Some are removed or destro in the process of "biopsy," while the evidence is more in keeping with a reg sive process in other lesions. A dysplastic change observed in a gravid wom may regress or even disappear several months following delivery, altho other lesions persist and show progression. Since "biopsy" in itself may a the course of these lesions, it cannot be assumed that dysplasia would ei disappear or regress had the lesions not been disturbed by the removal of sue for study.

Persistence for varying periods of time characterizes about 30 per cent of lesions now under study. During this interim, there may be periods of prog sion or regression. The changes may persist without appreciable change their severity despite multiple punch biopsies on numerous occasions, and called "sharp knife conization," in itself, does not invariably remove the en altered focus.

In about 10 per cent of the cases studied to date, a change interpreted t carcinoma *in situ* has ultimately been found. In none of these cases ca be established beyond all doubt that *in situ* cancer was not present w dysplasia was initially demonstrated. On the basis of cellular studies, howe there is suggestive evidence that dysplasia antedated the development of *situ* cancer in some instances. Only one patient has developed invasive c cer.

On the basis of experimental evidence, cellular and histopathological changes nalogous to those of dysplasia in the human are observed as the earliest orphological evidence of carcinogenesis in the mouse cervix. The cell shown FIGURE 8 is representative of those observed as early as four weeks in the aginal secretion of mice having a carcinogen impregnated thread inserted into he cervical canal as originally described by Murphy.[13] The changes are ini-ally demonstrated in large cells and include nuclear enlargement, hyper-hromatism, altered chromatin patterns, and cytoplasmic vacuolization. hese changes usually antedate the development of invasive cancer in the ouse cervix.

Conclusions

The term dysplasia can be used to designate a group of heteroplastic lesions volving the surface epithelium of the uterine cervix that are basically charac-rized by an increase in the number of primitive cells and by certain abnor-alities of cellular differentiation. In general, the lesions are less anaplastic han those which, in the opinion of the authors, warrant an interpretation of rcinoma *in situ.*

The altered cells removed from the dysplastic epithelium reflect the under-ing pathological change. The cells are predominantly polyhedral in form, ith relatively large nuclear masses, hyperchromatism, and, basically, a finely ranular chromatin pattern. The altered elements are usually isolated, but ay be arranged in sheets and only rarely occur in syncytial masses. Asso-ated changes of hyperkeratosis or parakeratosis are common.

At least some of the dysplastic changes may be associated with carcinogenesis the cervix. As evidence of this, comparable changes are observed, as the rliest cellular evidence of carcinogenesis in the mouse cervix and 80 per cent the *in situ* cancers in the human cervix show a coexisting focus of dysplasia. nce the latent period involved may be in excess of 10 years, a very protracted llow-up study is necessary before any definite conclusions can be drawn as the significance of the change in the human.

Acknowledgment

The authors are indebted to Doctors Roger B. Scott, Paul R. Zeit, and onald E. Hubbell for information regarding follow-up studies in dysplasia, d to the technical personnel in the Department of Cytology of the University ospitals of Western Reserve University for their cooperation in making this udy possible.

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UNUSUAL PATTERNS OF SQUAMOUS EPITHELIUM OF THE UTERINE CERVIX: CYTOLOGIC AND PATHOLOGIC STUDY OF KOILOCYTOTIC ATYPIA

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Much of the fascination of the study of exfoliative cytology of the uterine ervix results from the fact that one can observe cellular abnormalities that re not obvious on histologic examination of tissues. The insistence of cy- ologists on correlating morphologic cellular changes with tissue patterns has ndoubtedly been a factor in directing the attention of pathologists toward w-grade alterations of cervix epithelium that were hitherto little known and ither passed unnoticed or were labeled, without due consideration, chronic ervicitis, and thus thrown into the diagnostic waste basket of gynecologic athology. The difficulty of correct classification of such tissue changes has een well known for years. It was frequently circumvented by the creation f special diagnostic classifications such as the famous "rubrics" of Hinsel- ann.[7] On many occasions, new names have been created for groups of sions of unknown standing. Thus the terms anaplasia,[15] atypical hyper- lasia,[12] dysplasia,[4] precancerous metaplasia,[2] restless epithelium,[6] to name a w, were attached to poorly defined cervix lesions.

Inevitably, over a period of years, we have encountered a number of cases ifficult, if not impossible, to classify on histologic grounds.

As a result of long-term study, it appears that one and, possibly, a second issue pattern of disorderly epithelium can be isolated among these lesions. he first one is characterized essentially by the presence in histologic sections f large cells with relatively small but irregular and hyperchromatic nuclei urrounded by clear and transparent cytoplasm. Thus, the nucleus seems to e suspended in an empty space. For descriptive purposes, we have coined he term *koilocytotic atypia*, from the Greek word "koilos," meaning hollow or avity, to designate this lesion. Many of these lesions were recognized pri- arily in smears before their recognition in tissues. The term "warty dys- eratosis" was commonly used in the pathology laboratory when referring to is type of epithelial change.

The second atypical pattern, which will be mentioned only briefly in the resent paper, is not well defined and is characterized by the presence of ab- ormally elongated, spindly cells. Therefore, the term *spindly atypia* will be sed to describe it.

Review of the literature reveals that the koilocytotic atypia has been no- ced and illustrated by others, without any attempt, however, to isolate it as pathologic entity. Thus, Ayre[1] uses photographs of several of these lesions illustrate his precancer cell complex. Wespi[14] calls it irregular epithelium. apid and Goldberger[9] call it atypical cervix lesion. Reagan *et al.*[12] call it ypical hyperplasia or dysplasia of slight degree. Von Haam[4] calls it dysplasia. All of these authors consider it a tissue pattern that is below the le of obvious carcinoma.

The purpose of this paper is to present a series of cases of koilocytotic aty a pathologic and cytologic pattern that we believe to be reasonably well lined. The findings will be compared with *in situ* carcinoma and invas carcinoma of the uterine cervix. The possible significance of the koilocyt pattern in the genesis of cervix cancer will be discussed.

Material

Sixteen cases of koilocytotic atypia were recognized and studied. All th cases were discovered among the examinees of the Strang Cancer Prevent Clinic on routine cytologic examination of cervical and vaginal smears.

The youngest patient in this group was 28 years old at the time the les was detected, the oldest 50 years. The average age for the entire group 38.5 years, which is lower than the average age of 46.9 years for *in situ* ca noma as reported from our laboratory.[13]

Extensive biopsy material was available on all 16 cases and was arbitra classified according to the degree of tissue change. Thus, 4 cases of sligh cases of moderate, and 4 cases of marked koilocytotic atypia were recor

In addition to the above 16 cases, 4 additional cases were studied in wh the koilocytotic atypia was associated with the previously mentioned atyp spindly pattern of cervix epithelium.

Furthermore, 40 cases of *in situ* carcinoma and 110 cases of invasive cerv cancer were examined. Cytologic as well as pathologic material was exami on all of the cases of *in situ* carcinoma. Smears and pathologic material w investigated in 10 cases of invasive carcinoma. In 3 of these, *in situ* carcin was still present. The remaining 100 were cases of stage 1 cervix cancer fr the files of the department of pathology. On the average, 4 slides of cer lesion were available on each case. In 50 of these cases, the surface was cerated and, in the remaining 50, there was the concomitant *in situ* and in sive pattern. Thus, there was a total of 53 invasive cancers with *in situ* co ponent. The latter cases constitute the baseline and checking point of material. The material was examined with emphasis on the following poi (1) cytologic and pathologic criteria of atypical koilocytotic patterns of cer epithelium; (2) relationship of the koilocytotic lesions with other forms atypia and with *in situ* and invasive carcinoma.

Owing to the courtesy of Walter Tolles of the Airborne Instruments Labo tory, Inc., Mineola, N. Y., accurate measurements of cellular compone could be made in several of our cases.

Cytologic Findings

All cytologic observations reported below were made on material stai according to the method of Papanicolaou.

Cytologically, all the cases of koilocytotic atypia were characterized by predominance of atypical cells approaching the size and general appeara of superficial and parabasal cells of the squamous epithelium.

The striking features of such cells were (FIGURES 1, 2, 3 and 4):

(1) Abnormal, moderately enlarged, and hyperchromatic single or multiple uclei. The degree of hyperchromasia varied from slight to marked. The ajority of the nuclei displayed a granular structure of chromatin, but some ook the stain in a diffuse, dense manner obscuring structural details. Al-ough occasionally irregular in shape, the nuclei were usually sharply out-ned.

(2) Abundant cytoplasm that was predominantly basophilic, although sinophilic cells were not uncommon. It appears to the authors that, in

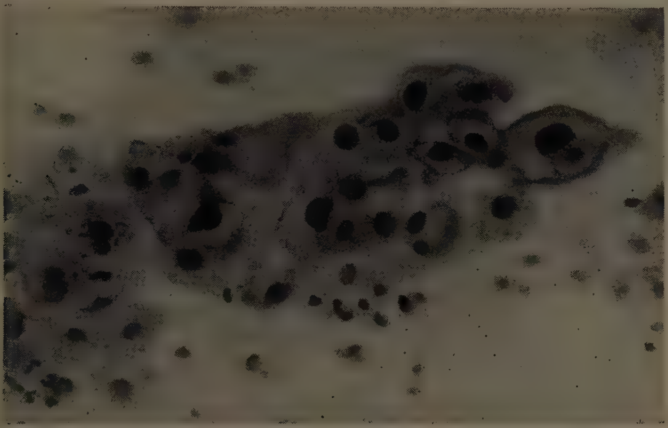


FIGURE 1. Cluster of cells characterized by slight nuclear hyperchromasia which is emphasized by the pres-ce of a clear perinuclear zone. The occurrence of such cells in clusters is not uncommon in the koilocytotic ypia. ×300.

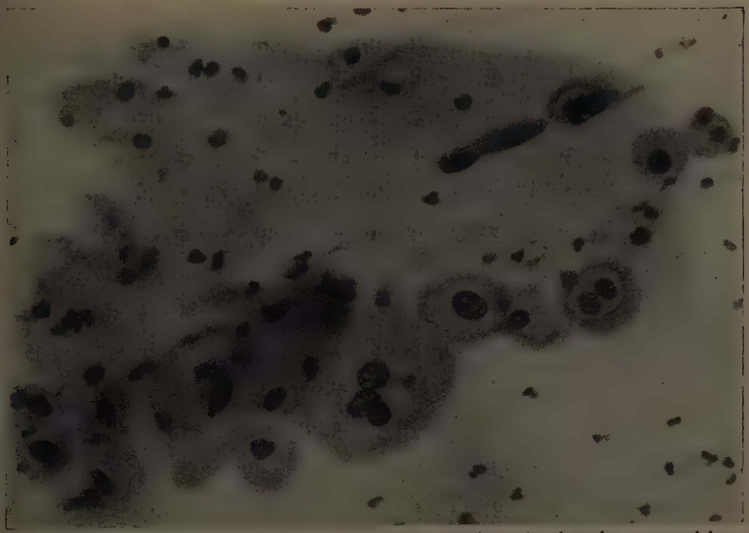
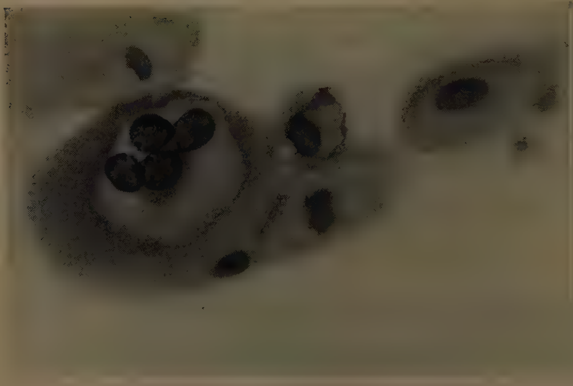


FIGURE 2. Cluster of cells showing clearly the contrast between the perinuclear clear zone and denser periph-l cytoplasm. Multinucleation is evident as well as some anisonucleosis. ×300.



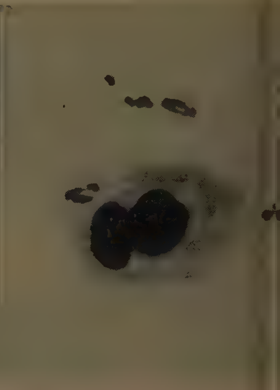


Figure 3 (left). Detail of the preceding photograph. The perinuclear zone and multinucleation are cle seen. ×480.

Figure 4 (right). Cell occurring in koilocytotic atypia. Hyperchromasia is more accentuated in the la double nucleus. The perinuclear zone is clearly noticeable. ×480.

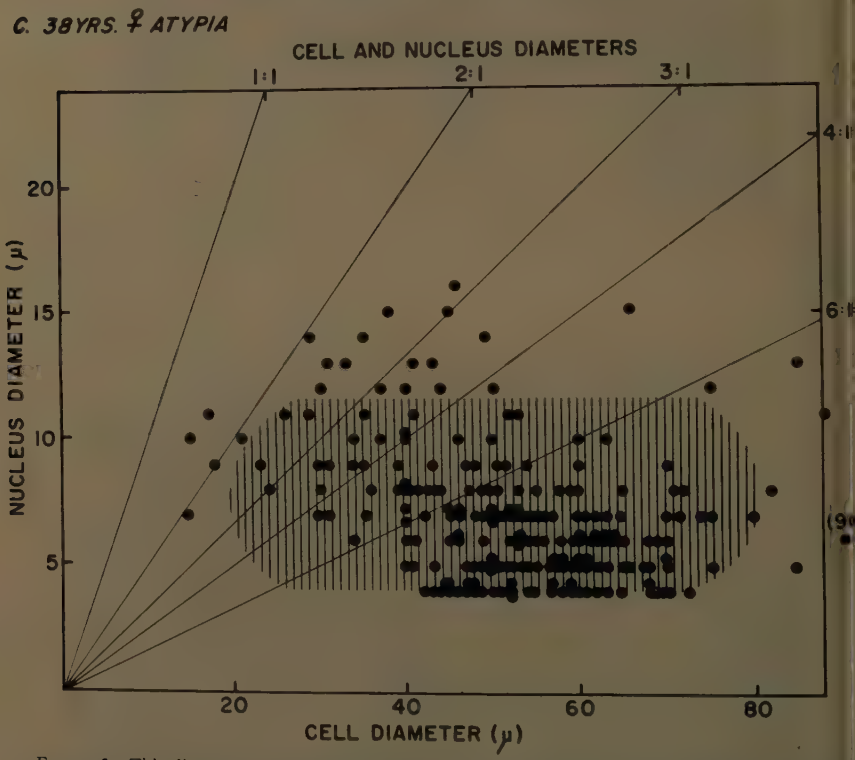


Figure 5. This diagram was obtained by plotting cell diameter against nuclear diameter. The cen striated portion of the diagram represents average area occupied by plotting these dimensions in a healthy menopausal woman. It will be seen that in koilocytotic atypia, the atypical cells falling outside the stria area are relatively large, and that their cytoplasmic:nuclear diameter with few exceptions is higher than A few cells, however, fall into the 1:1 and 2:1 area.

nost instances, the increase in the amount of cytoplasm paralleled nuclear nlargement. Moreover, the cytoplasm was not uniform and presented one eature that is most characteristic of the cells under discussion, namely:

(3) Perinuclear zones that were distinctly different from the peripheral ytoplasmic areas. In some cells, the perinuclear zones were clearly outlined nd transparent, obviously consisting of a very thin layer of cytoplasm. In ther cells, the perinuclear zones were less transparent, taking the stain just shade lighter than the peripheral cytoplasm. Variations in thickness of the erinuclear zone, at times, were also noticeable within one cell, the thinnest ortion being always perinuclear in position. In some cells, either segments r the entire perinuclear zone contained yellow staining material which, in apanicolaou stain, is presumably glycogen. This impression was partly ubstantiated by staining histological sections of a few cases of koilocytotic typia with periodic acid-Schiff reagent controlled with diastase incubated naterial. Traces of glycogen were demonstrated in the cytoplasm of some f the large cells present in the tissue lesion.

The area occupied by the perinuclear zone varied in size and in position. n most cases, the clear zone occupied one fourth to two thirds of the total

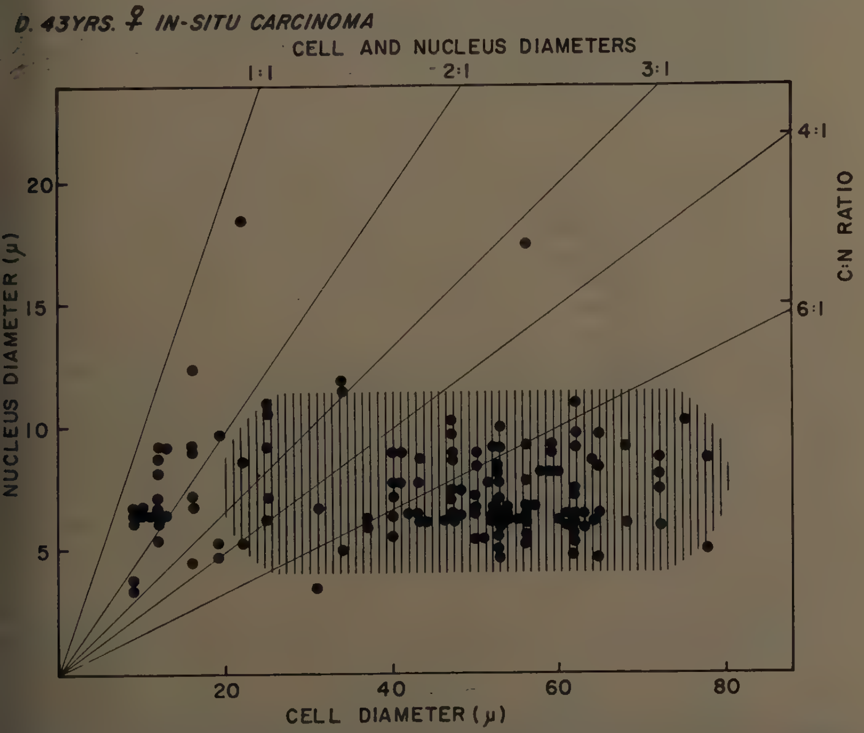


FIGURE 6. Similar diagram computed for a case of *in situ* carcinoma. It is evident that there is a tendency cell grouping in the area of lower cytoplasmic:nuclear ratio or a "shift to the left."

cytoplasmic surface, but sometimes it was so wide that only a rim of thic peripheral cytoplasm remained. For the most part, the perinuclear zone w centrally located but, occasionally, it was eccentric in position. In these lat cases, the nucleus was also eccentrically located. The significance of the pe nuclear zones is not clear at this point. It is possible that the zones res from altered cellular metabolism.

In three cases of moderate and in two cases of marked koilocytotic atyp the above described cells were supplemented by a few small cells with atypi nuclei and scanty cytoplasm. By our standards, these cells were morpholo cally indistinguishable from cancer cells.

Accurate measurements of cell and nucleus diameter performed by t Airborne Instruments Laboratory, Inc., indicate that the marked koilocyto atypia is characterized by the presence of exceptionally large abnormal c with a high cytoplasmic:nuclear ratio. The presence of a few smaller abnorm cells with decreased cytoplasmic:nuclear ratio may also be noted (FIGURE It is of interest to compare this diagram with that of *in situ* and invasive cer carcinoma (FIGURES 6 and 7). It will be observed that the measurements

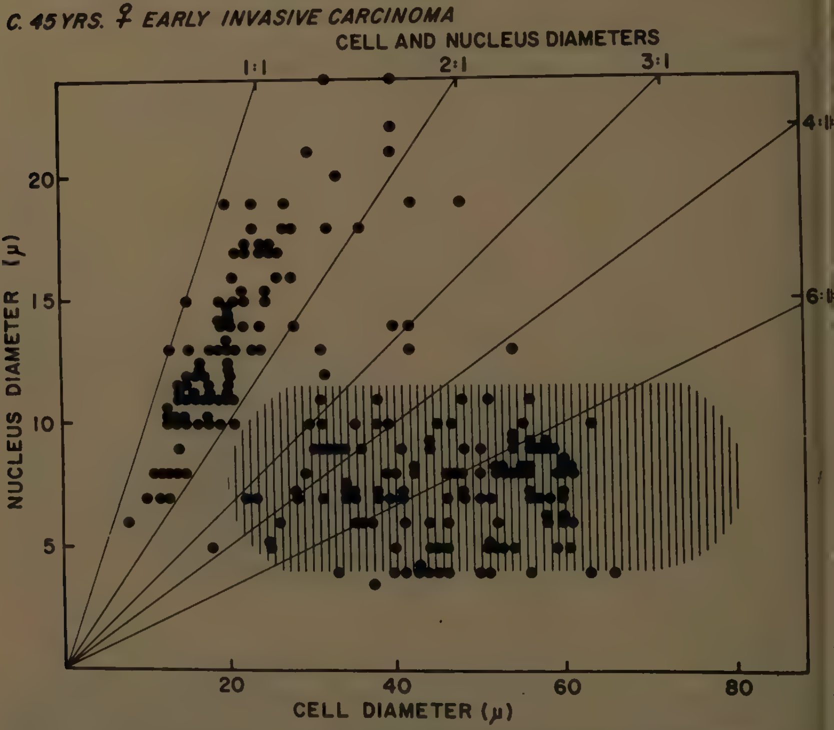


FIGURE 7. Similar diagram in a case of invasive carcinoma with associated *in situ* component. The "s to the left" is even more marked than in the preceding diagram. FIGURES 5, 6, and 7 represent modificatior data obtained through the courtesy of the Airborne Instruments Laboratory, Inc.

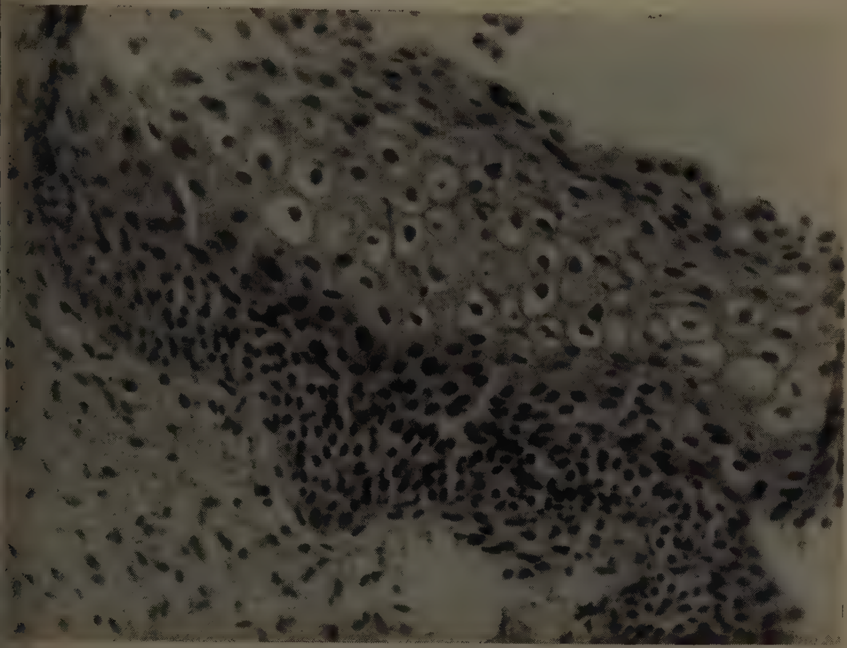


FIGURE 8. Minimal koilocytotic atypia. The epithelium contains, in its upper half, somewhat enlarged cells ith clear perinuclear zone and slight nuclear hyperchromasia. Note parakeratosis on the surface. ×300.

mall cells seen in association with koilocytotic atypia fall well within the range f cancer cells with cytoplasmic:nuclear ratio between 1:1 and 2:1.

Pathologic Findings

Histologically, all 16 cases under discussion represented tissue changes below ne level of obvious carcinoma. In four of our cases, only a minimal tissue sion was found (FIGURES 8 and 9). This lesion consisted of a focus of large, ear, apparently vacuolated cells located at the squamocolumnar junction or ithin the squamous epithelium of the portio. The individual cells composing ne focus were comparable with the abnormal cells seen in smears and, obvi- usly, were not the result of an artifact. The focus was fairly sharply circum- ribed and, usually, it was located at the surface of the epithelium. In the nderlying and surrounding epithelium, there was no evidence of any abnormal tivity, particularly of mitotic activity. A few layers of flattened parakera- otic cells usually covered the lesion. It is reasonably certain that if it were ot for the cytologic findings these minimal lesions would have passed unno- ced.

In eight cases, the lesion was more extensive and was classified as moderate IGURES 10, 11 and 12). In these cases, the abnormal large cells were present one-half to three-fourths of the superficial portion of the epithelium. There

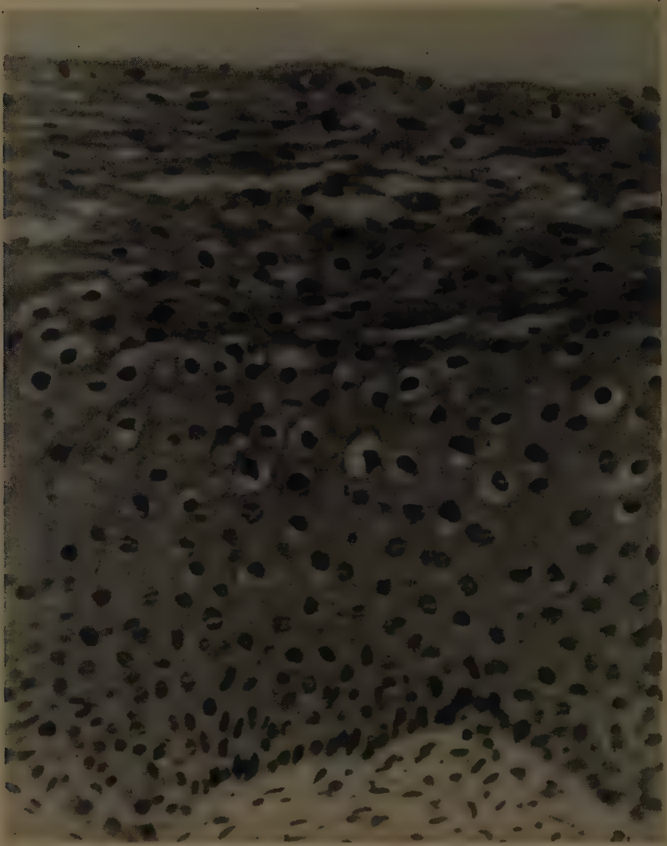


Figure 9. Essentially similar tissue pattern to figure 8. Parakeratosis is more prominent. ×300

was usually a somewhat greater variability in the cellular and nuclear s
not only of the large clear cells, but also of the intervening epithelial cells.
a few cases, the lesion was composed of an accumulation of large clear c
only. Infrequently, some proliferation of the basal layer was noted. In
cases, however, the basal layer was essentially orderly, although an occasio
mitotic figure was present. Scattered dyskeratotic cells were usually no
within the lesion. In three cases, the surface of the lesion was covered w
a thick layer of flattened parakeratotic cells.

In four cases, the koilocytotic atypia was marked (figures 13, 14, 15,
and 17). The lesion was found within the cervix in three of these cases.
the fourth case, the lesion was located in the vagina and occupied the are
a hysterectomy scar. The essential features of the epithelial changes w
similar to those described above, except that the lesion was more disorde
and occupied practically the entire thickness of the epithelium. Large c
were still very conspicuous, yet the intervening epithelial cells were less regu

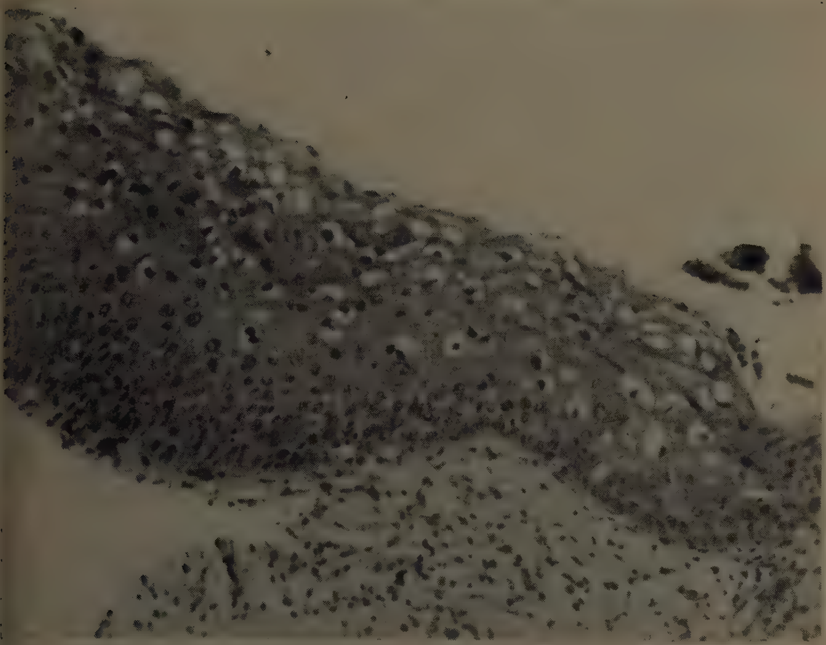


Figure 10. Koilocytotic atypia of moderate degree. Note large cells with perinuclear zones in the upper lf of the epithelium. Multinucleation is clearly seen. ×150.

nd there was obvious nuclear enlargement and hyperchromasia. In all four ses, a concomitant epithelial pattern characterized by disorderly arrange-ent, and increased hyperchromasia of cells of all layers was noted. This tter pattern was of quite suspicious nature and bordering on *in situ* carci-oma. In one case, there was extension of the suspicious epithelium into an ndocervical gland and, in another case, so-called tripolar mitoses were noted. he latter finding was originally described by Parmentier and Dustin[11] as oc-rring in *in situ* carcinoma of the uterine cervix. These abnormal mitoses e characterized by the presence of two polar groups of chromosomes, in addi-on to the equatorial plate in the metaphase. Hamperl *et al.*[5] confirmed the esence of such mitoses in *in situ* carcinoma, and we also have found them some, but not all, of our own cases.

In four additional cases, as mentioned above, moderate koilocytotic atypia as associated with a still different form of atypia of cervix epithelium. The sociated lesion was composed essentially of elongated cells arranged in a sorderly fashion in the central area of the epithelium but parallel to the rface in superficial portions. The presence of multinucleated, abnormally yperchromatic cells was noticeable. A fairly orderly basal layer, along with rface parakeratosis, completed the picture (figures 18 and 19). Although is spindly epithelial pattern is of a very suspicious nature, we hesitate at

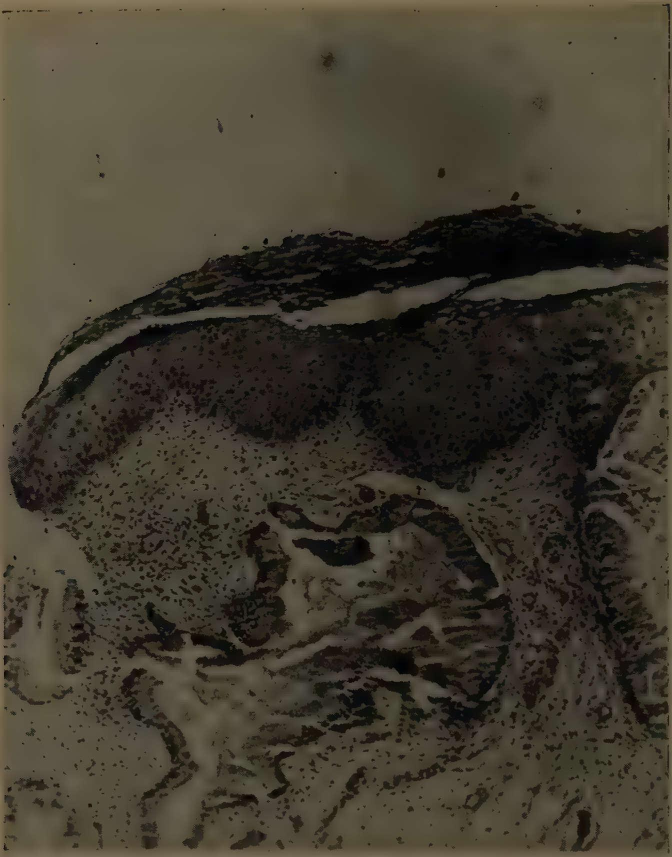


FIGURE 11. Koilocytotic atypia of moderate degree. Note pronounced parakeratosis and the presenc
cells with perinuclear vacuoles. ×77.

this point to call it outright *in situ* carcinoma in the absence of an associat
more classical pattern.

Koilocytotic Atypia and Cervix Cancer

In order to investigate the possibilities of the association of koilocyto
atypia with classical patterns of cervix cancer, we studied, cytologically a
pathologically, 40 cases of unquestionable *in situ* carcinoma of the uter
cervix. In 5 cases, we noticed, in smears, a fair number of cells characterizi
koilocytotic atypia. In 4 of these cases, the koilocytotic pattern was prese
in tissues in association with *in situ* carcinoma. In the fifth case, no sou
of these cells was determined on the material available. In 13 additional cas
isolated cells of similar nature were noted in the smears. In 6 cases, exami
tion of tissues revealed, in association with *in situ* carcinoma, the presence

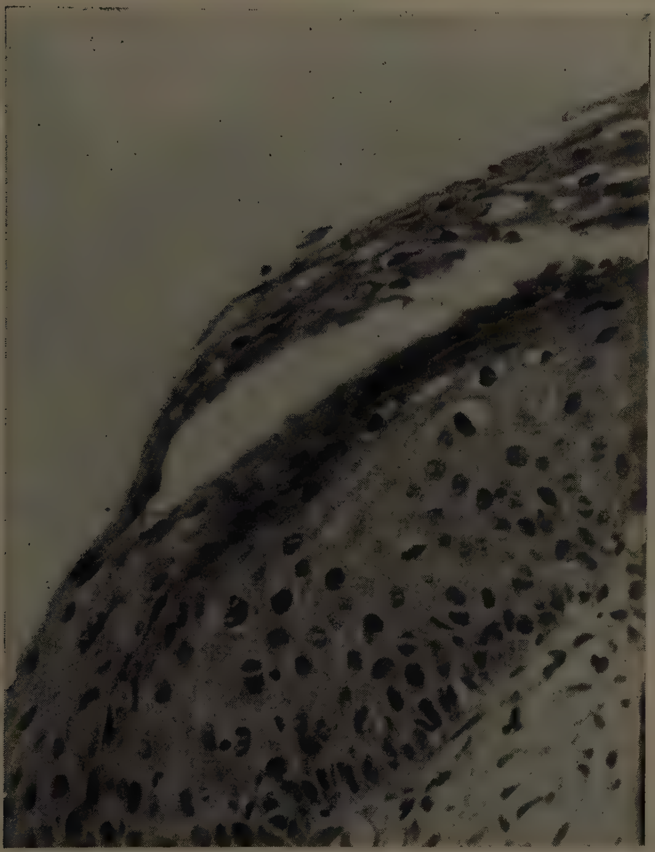


FIGURE 12. Detail of FIGURE 11. Note moderate nuclear atypia and the presence of a mitotic figure. ×300

an epithelium containing a few isolated large, clear cells somewhat suggestive of koilocytotic atypia. In the remaining 7 cases, the only possible source of atypical cells was the surface cancer, which contained large pseudovacuolated cells similar to those seen in koilocytotic atypia.

Cytologic investigation of 10 cases of invasive carcinoma revealed that this type of cellular atypia was encountered only in rare instances and consisted of a few isolated cells.

Pathologic investigation of 110 cases of invasive carcinoma gave the following results: in 57 cases of invasive cervix cancer with ulcerated surface, there was no evidence of koilocytotic tissue pattern. Although cancer cells with clear cytoplasm were not uncommon, the degree of nuclear change in these cells was far greater than anything seen in our atypia cases. In 53 cases of invasive cervix cancer with *in situ* carcinoma still present, clear-cut koilocytotic atypia was noted in three cases adjacent to, or in continuation with, *in*

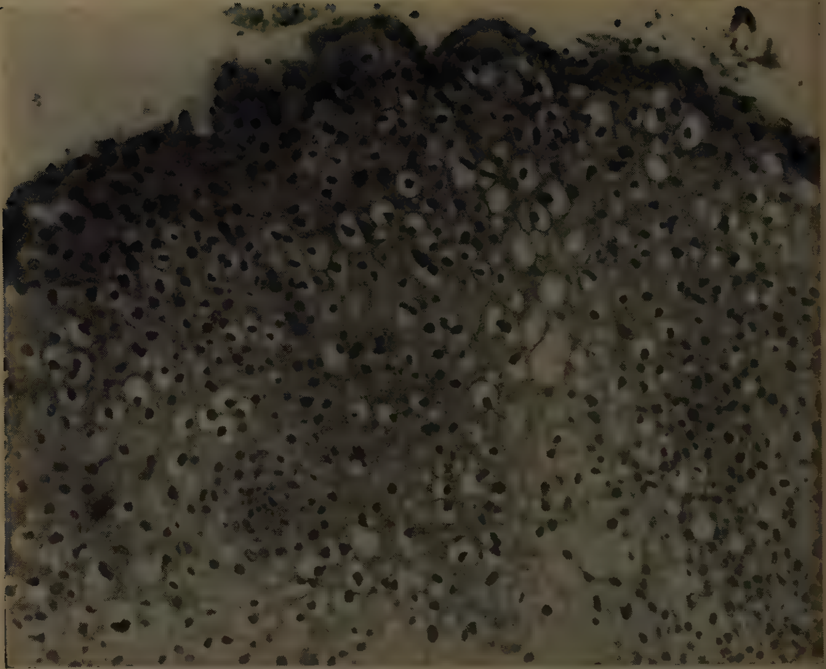


FIGURE 13. Marked koilocytotic atypia. The entire thickness of the epithelium is composed of the chara teristic cells. ×150.

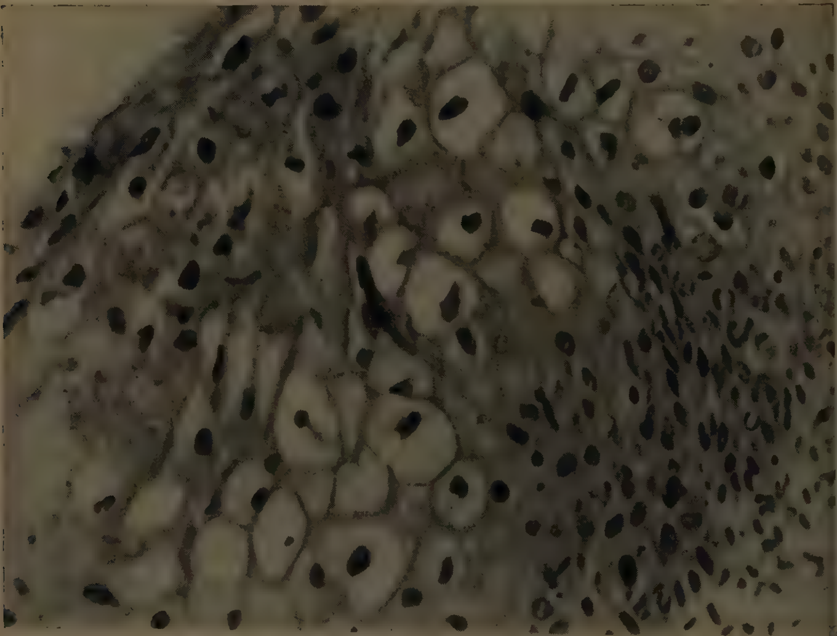


FIGURE 14. Detail of FIGURE 13. ×480.

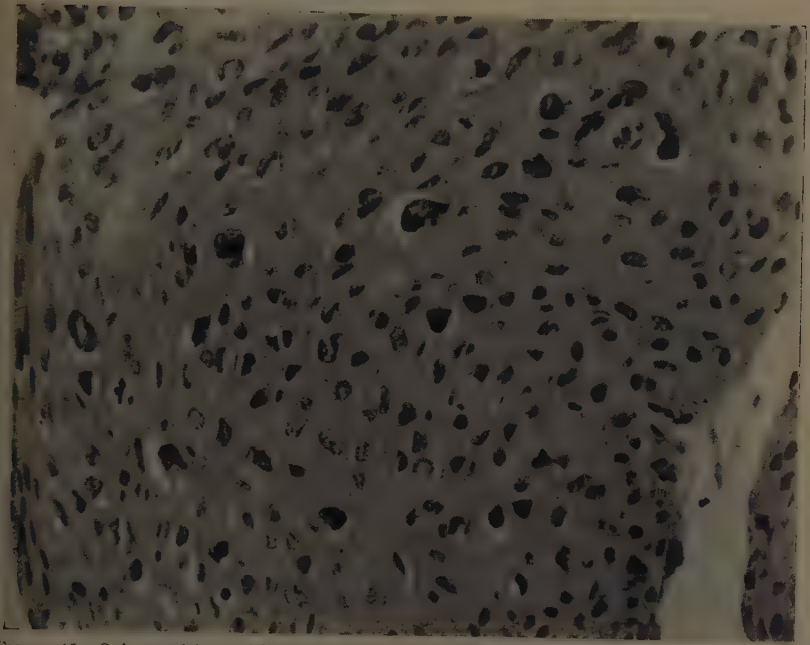


FIGURE 15. Quite suspicious epithelial pattern found in association with that pictured in FIGURES 13 and 14. Note marked nuclear abnormalities. ×330.

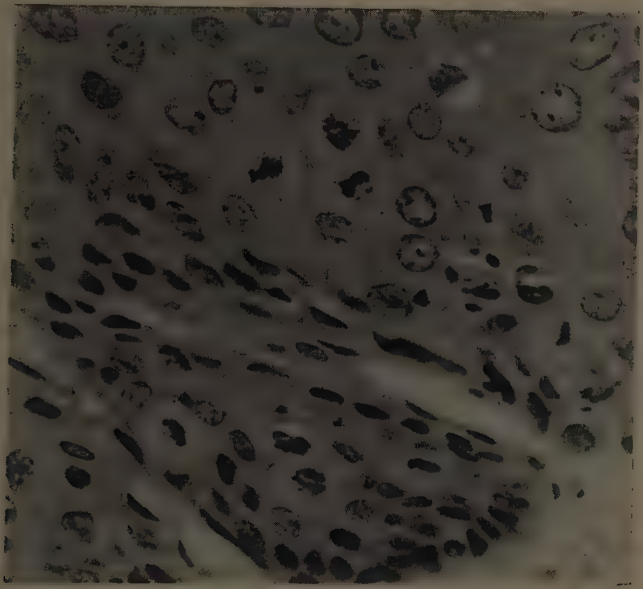


FIGURE 16. Atypical mitosis in another area of same section as shown in FIGURE 15. ×480.

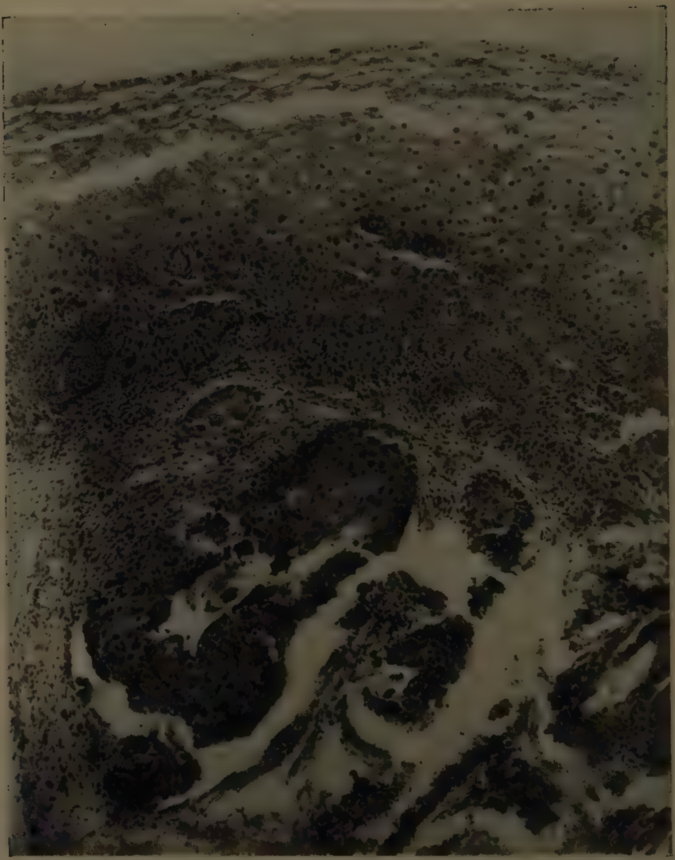


FIGURE 17. Marked koilocytotic atypia of surface epithelium associated with the presence of a different an
very atypical epithelial pattern within an endocervical gland. ×77.

situ. In all three cases, there was evidence of marked proliferation of the basa
portion of the koilocytotic lesion. The pattern of invasive cancer in thes
three cases was that of the average medium-sized cell variety of epidermoi
carcinoma. In six additional cases of invasive carcinoma with *in situ* com
ponent, there were areas of surface epithelium with slight degrees of atypi
somewhat reminiscent of the koilocytotic pattern.

Thus, the cells and tissue patterns described in the first part of this paper a
characterizing koilocytotic atypia may occur in association with cervix cance

We have also investigated, cytologically and pathologically, a number c
unquestionably benign cervix lesions, including a large series of squamou
metaplasias of the endocervix and a few papillomas of the portio. In fou
cases of somewhat atypical squamous metaplasia and in two cases of benig
papillomas, we noticed, in smears, the presence of cells with perinuclear zone

Therefore, the presence of such cells not only fails to rule out cancer but

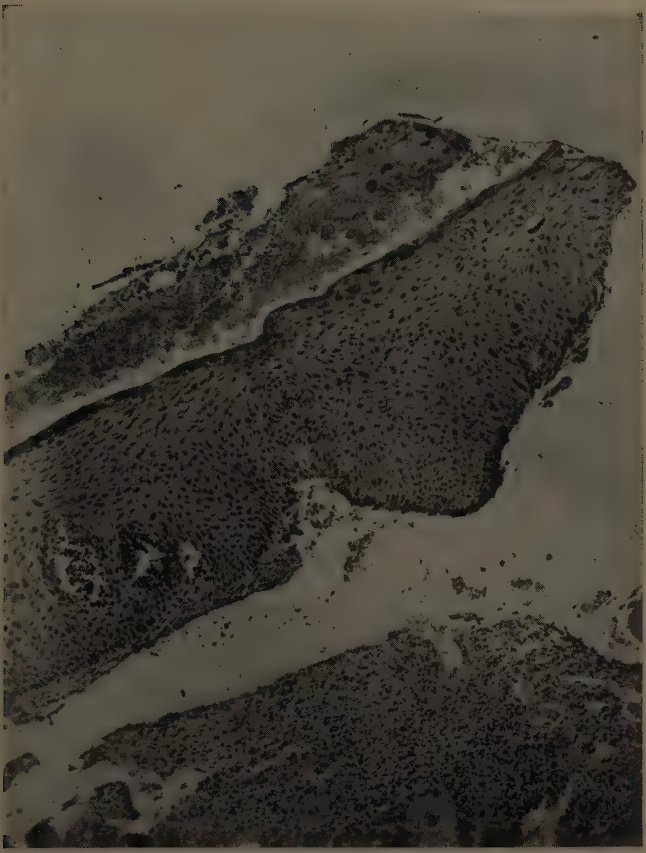


FIGURE 18. Spindly pattern of epithelial atypia. ×77.

is, by itself, insufficient to determine accurately the type of tissue lesion. In the final analysis, we must rely on pathologic investigation.

The cellular abnormalities characterizing koilocytotic atypias, of course, have been noticed before. They fall into Papanicolaou's category of superficial, intermediate, and parabasal cell dyskaryosis.[10] This classification of abnormal cells is of didactic value only, since pathologic findings clearly indicate that large atypical cells may be found at all levels of the epithelium.

Ayre has included similar cells in the "precancer cell complex." This term carries serious prognostic implications. In our experience, however, at least some of the koilocytotic lesions have a tendency to regress. Held[6] also expresses doubts as to the invariably neoplastic nature of the "precancer cell complex." In a series of 12 similar cases presented by Lapid and Goldberger,[9] the lesion disappeared spontaneously in two patients; one patient developed carcinoma; and nine remained unchanged over a period of observation varying between nine months and four years. Papanicolaou[10] also speaks of reversi-

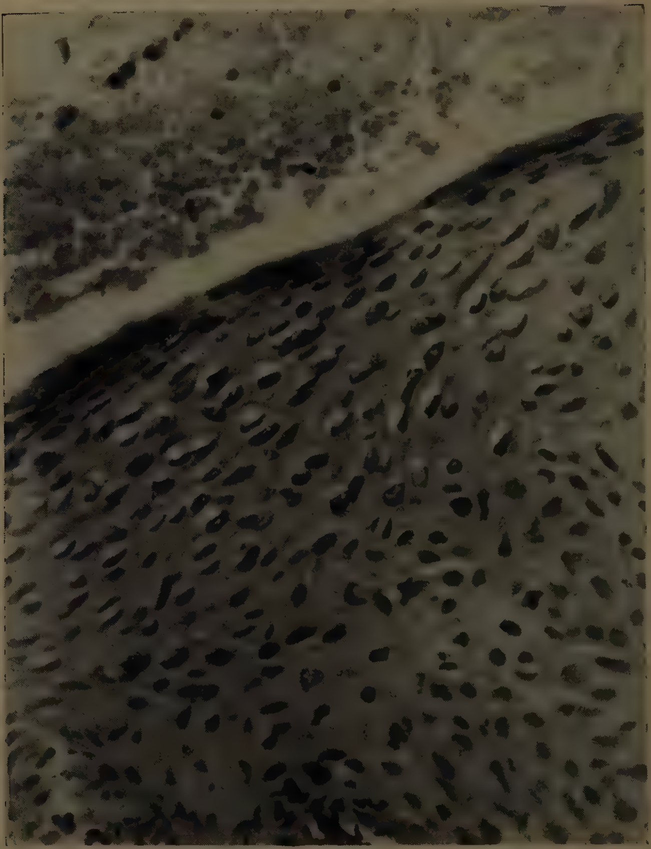


FIGURE 19. Spindly pattern of epithelial atypia. Detail. ×300.

bility of the dyskaryotic patterns, and emphasizes that this happens mo
frequently with cells of the superficial and intermediate type than with dy
karyotic cells of other types. Finally, in Reagan's experience,[12] many of th
atypical cervix lesions disappear. There is little doubt that the koilocytot
atypias are among those lesions.

Conclusions

The koilocytotic atypia is an alteration of the surface squamous epitheliu
of the cervix and, when uncomplicated, it cannot be called cancer by our pre
ent criteria. The recognition of this epithelial pattern by pathologists w
undoubtedly clarify the origin of many an abnormal cell seen in smears. Koil
cytotic atypia can be associated with *in situ* or invasive cancer of the uteri
cervix. Therefore, recognition of this pattern, either in smears or in sectio
should not preclude search for cancer. The role of koilocytotic atypia as
stepping stone in the genesis of cervix cancer is not clear and, at this poi

we cannot exclude the possibility that some of these lesions may progress to cancer. The evidence accumulated so far, however, indicates that some of these lesions may regress. At any rate, it is unlikely that the koilocytotic lesion is a part of the natural history of more than a fraction of cervix cancers. In our own experience with a small number of women who developed *in situ* carcinoma while under observation,[8] no clear-cut cytologic abnormalities suggestive of koilocytotic atypia were observed prior to the appearance of cancer. We feel that a great many years of follow-up will be necessary before the nature and the behavior of koilocytotic atypias and related lesions of cervix epithelium will be determined accurately.

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THE PRECANCER CELL: A NEW PROBLEM IN CANCER RESEARCH*

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Rather than speak of the precancer cell as a new problem in cancer researc perhaps we should refer to it as a "new opportunity" or new method of co ducting functional research in the living subject. It is not the purpose of th paper to present the results of a concluded experiment but, rather, to prese the broad panorama of research work still in progress utilizing this uniq approach.

The introduction of exfoliative cytology into contemporary medicine Doctor George N. Papanicolaou has been the springboard for new advanc in techniques for early diagnosis. Of equal importance, though slow to applied, is the new research approach to cancer of the cervix. It has no been widely recognized that the cervical smear or cell scraping will reveal wit great accuracy the presence of carcinoma *in situ* in cervical tissues that ma appear benign or completely normal to the naked eye. For some time, th was considered the earliest recognizable stage of cervical malignancy. T use of cervical cytology for routine cancer screening, however, especially whe the surface cell biopsy technique is used, has revealed an in-between stage whe the cells are neither normal nor malignant in appearance. These cells we reported by our group after the introduction of the cervical spatula (1946 and were described as the "precancer cell complex." This cell entity has r mained highly controversial in diagnostic practice. Its importance is great in basic research, however, because of the opportunity afforded to explo carcinogenic influences and to examine the cell-transformation stage fro benign to anaplastic. The advantage of the scraping technique in researc is the consistency of the sample in serial cell studies. If one depends on phys ological exfoliation, on the other hand, many variable factors influence t number of anaplastic cells per specimen; for example: the volume of cervic mucus, the point of collection of specimen, and the technique used. As result, individual specimens will vary between positive, suspicious, doubtfu or atypical.

Exploration by the research cytologist of this developing neoplasm enabl precision studies of cell behavior and cell response before the potential carc noma reaches the dangerous stage. It is possible to trace premalignant chang back to their normal cell precursor, and true behavior patterns of regressio progression, and latency reveal the full duration and life history of epidermoi cancer.

Clinical observers are provided with a powerful new research tool to e plore the developing neoplasm and its precancer cell components before the is tissue breakdown or necrosis to complicate chemical or cytochemical invest

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* This work was supported by a grant from the Public Health Foundation for Cancer and Blood Pressure R search, Inc., New York, N. Y.

gations. During behavior study, it is possible to observe and record variations in exfoliation of precancer cells during various stages of the sex cycle, and to determine at close range the changes in abnormal cells, if any, caused by the superimposition of pregnancy when normal and when abnormal. We have observed that abnormal pregnancies culminating in abortion, or those of ectopic type, exert a more profound stimulating effect upon the premalignant cell than normal pregnancy. During our 10-year experience in studying cell behavior, we have failed to observe favorable regressive effects of pregnancy upon premalignant cells. Pregnancy is known to influence cervical tissues by causing cervical hypertrophy and hyperplasia accompanied by increased outpouring of cervical mucus. Premalignant or carcinoma-*in situ* lesions of the cervix participate in the generalized hypertrophy and hyperplasia of pregnancy, and a small lesion grows in extent along with the rest of the tissues, possibly more so. It is our belief that cervical mucus may play some role as a growth stimulant, possibly due to its estrogenic content (possibly, too, as a result of other pregnancy hormonal influences). There are undoubtedly defense mechanisms in operation. We have not seen evidence that the pregnancy *per se* will stimulate growth of the *in situ* lesion sufficiently to cause infiltration. We have observed further that, during the postpartum period, premalignant lesions undergo involutional changes along with the cervix as a whole. The reduced extent of the lesion and the diminished number of cells recoverable during such periods may account for the belief, which we consider erroneous, that such lesions frequently undergo spontaneous regression during or after pregnancy. If careful cell scrapings are secured from such cervices, small nests of the premalignant or *in situ* cells, in our experience, will be revealed consistently, and we assume that at some future time progression to a more advanced stage is likely to occur.

Effects of Estrogen

Estrogen, when administered artificially, whether in small or large doses, will tend to stimulate growth of premalignant lesions. As a result, increasingly large numbers and increasingly bizarre morphological cells will be found during estrogenic administration. Furthermore, we have observed unusual cell changes, as in cells that appear vermiform or snake-like in appearance, in which the cytoplasmic structure would seem to have been radically altered. Such cells appear as if they have been ballooned up and then become deflated or flaccid. While the cytoplasm appears to have lost something, the nuclei seem to have increased in size and density, showing evidence of increased hyperchromatism. Precancer cells under these conditions pile up and are thrown off in profusion. At the same time, the normal cervical cells show evidence of maximal cornification, as would be expected with continuous estrogenic stimulation. While we have observed some favorable changes in inflammatory and metaplastic cervical lesions following estrogenic therapy, we have observed no evidence of regression of premalignant or *in situ* lesions during or following estrogenic hormone administration.

The Training of the Research Cytologist

The opportunity for profitable research studies in the cervix is so great i our opinion as to justify special training of the research cytologist to includ exhaustive correlative clinical, cytological, and histological studies aimed a the development of experienced judgment in all three fields. As his trainin approaches completion, the cytologic studies become of dominating importanc and the clinical and the histological come to assume a secondary and merel confirmatory position. Following such meticulous training, one may prognosticate tissue findings on the basis of cell scrapings with considerable accuracy.

Surgical confirmation of all positive cytology is constantly advised in diagnostic practice. Research, on the other hand, should be done under ideal conditions, and rules of research, rather than those of diagnostic practice, shoul apply. Any surgical interference whatsoever may impair or completely nu lify conclusions relative to cell behavior or cell response. But a remarkabl opportunity to use the premalignant cells as a testing board for chemicals o antibiotics administered locally or systemically is provided by the highly sens tive cytologic mirror.

Before presenting some observations made during this 10-year experimentation on the nature of the precancer cell complex, I should like to explai that we are not in a position to present a population study or a complete statistical report since, in many cases, these studies are still in progress. W are in a position, however, to report on a comprehensive series of special cas studies, each of which represents a long-term experiment in cell behavior whic finally reaches a conclusive stage when complete histological studies are mad

Research Case Studies

While most of our behavior studies were conducted entirely on the basis o serial cytological examinations, the first special case studied proved to be or in which it was possible to do combined histological and cytological studie repeatedly throughout a three-year period. This was possible because th patient's lesion involved the entire squamocolumnar junction of the cervi rather than only one small segment, as is so frequently the case. It was po sible, therefore, to secure repeated punch biopsies without risk of disturbin or removing the neoplastic tissue from the other quadrants. This patien Mrs. G., was a young Jewish woman, aged 32, who was pregnant when th first cytological cell scraping revealed the presence of anaplastic cells of precancer cell complex type. Her first histological study was diagnosed as carc noma *in situ*. The pregnancy was allowed to progress, although repeated ce scrapings were taken each month in order to determine possible evidence o progression. Two months after the normal termination of pregnancy, cervic scrapings from the normal appearing organ still revealed the presence of th same type of cell. During the following two years, an intensive investigatio was undertaken on this patient, as follows:

The patient visited the Cancer Institute at Miami every one to two week for cytological and cytochemical studies. Tissue culture studies were mad at frequent intervals, during which the *in vitro* study of growth velocity was undertaken by C. G. Grand, our tissue culturist. Large quantities of the patient's cervical mucus were analyzed chemically for their steroid content using paper chromatography, and estrogen was shown to be continuously present in small quantities. Some of the mucus was preserved for viral studies using the egg inoculation technique, but this study proved inconclusive. A test of Aureomycin therapy in vaginal suppository was made, and a mild allergic reaction of the tissues was noted. Also, there was evidence of some temporary regression of the neoplasm, which recurred upon termination of therapy. During this two-year period, the patient had over 200 cervical cell scrapings and five surgical biopsies (punch), while fragments of tissue were removed for tissue-culture study on four separate occasions.

The patient became pregnant a second time and, following thorough consultation with several medical men, Doctor Joseph Scott, her gynecologist, decided to allow her to carry through with the second pregnancy. As the pregnancy progressed through the first trimester, the cell scrapings showed more abundant and more active cervical cells of carcinoma-*in situ* type, and at the fourth month, a ring biopsy was performed in an attempt to remove, if possible, all of the involved tissue. Histological studies at this time continued to show the pattern of carcinoma *in situ* with glandular spread. Cervical cell scraping, a month after the surgery, revealed small groups of anaplastic cells still present in the cervix. Monthly cell scrapings were taken throughout the pregnancy, and the cell findings continued to be quite active. There was no indication that infiltration of the neoplasm had taken place. At the eighth month of pregnancy, the patient was delivered of a healthy infant by Porro's cesarean section, and subsequent histologic studies of the cervical tissues again showed the same picture as earlier: namely, extensive carcinoma *in situ* with glandular invasion. There was no evidence of stromal infiltration, and it was the universal opinion that the *in situ* carcinoma had been completely eradicated and the disease cured.

This case illustrates several important points. First, carcinoma *in situ* does affect the Jewish woman at times, and when it does, it is not a transient disturbance. Second, there was no regression of the disease during or after pregnancy. Third, this case pointed out the safety of conducting long-term behavior studies of carcinoma *in situ* as long as frequent cytologic studies and occasional biopsies may be undertaken.

With the majority of our 339 cases included in the precancer cell progression study, no tissue diagnosis was made until the termination of the experimental study, which extended over a period of from six months to six years. While a complete breakdown of results is not yet available, 121 of these cases have reached a conclusive stage in that their cell behavior study was terminated by complete histological diagnosis. In 92 of these cases, the diagnosis of carcinoma *in situ* was made, while early infiltration was reported in 29.

It is our belief that regression of minimal precancerous or carcinoma-*in situ* lesions may follow surgical biopsy. This was observed in 18 cases. On the other hand, we have failed to observe *true* spontaneous regression in any cases included in this series during the 10-year period. Not all cases have show progression, however, as will be shown later.

Variable Velocity of Progression

One outstanding case in this series shows extended nonprogression throughout an eight-year period. At one stage, however, this patient showed moderat regressive changes, and cervical cell scrapings revealed no more than inflammatory features with precancerous tendencies. In order to determine whethe or not regression had taken place, estrogen was administered to this patient i the form of stilbesterol, 1 mgm. T.I.D., and within one month, the anaplasti cells of precancer cell complex type reappeared in large numbers, indicating tha the lesion was dormant but had not completely regressed.

In contrast to prolonged latency, numerous cases have been observed t show rapid progression. It appears that some cases do remain in a precancerous or *in situ* stage for prolonged periods of months or years. Others progress rapidly. Indeed, one of these cases that was studied carefully over 16-month period showed progression from normal cells throughout the las four months of pregnancy, with minimal precancer cells appearing at 19 day postpartum. This case progressed rapidly to a stage diagnosed as early invasive cancer during an 11-month period.

Origin of Cancer Cells Traced Back to Normal Cells

As a result of frequent annual screening of women for cervical cancer, it is now possible to link up the cancer cell and the premalignant cell with its norma precursor. At the Royal Victoria Hospital and McGill University, Montrea Canada, and, more recently, at the Cancer Institute at Miami, it has bee found possible to check back to previous cervical cell scrapings one, two, an three years prior to the appearance of the premalignant or *in situ* cancer ce in cytology smears. Nine cases have now been recognized in which cance *in situ* may be traced back to a normal cell stage while, in one case, early infi trating cancer also may be traced back to a normal cell precursor.

Research Cytology Screening of Antibiotics and Hormones

Once the research cytologist has clearly learned the type of anaplastic cervica cell that persists and does not regress spontaneously or with subsiding inflammation, it is possible to use such patients harboring the precancer cell comple as research subjects. Ideally, intravenous or systemically administered hormones or antibiotics might conceivably affect locally carcinoma-*in sit* lesions, causing regression. Thus a true specific cancerous cytotoxic influenc could be demonstrated. Unfortunately, in our experience, no such agent ha been discovered. Sarkomycin, a new Japanese antibiotic, however, has bee shown to have some antitumor influence causing temporary regression of a precancerous lesion that recurred after therapy was terminated. This appears t be a random response, since other cases were shown to have no response whatsoever.

An alternative method of administration is to use the vagina as a repositor for various antibiotics prepared in topical suppository form. As the suppository dissolves in the cervical secretions, it would appear that the chemical environment of the involved cells may be altered in such a way as to cause the selective destruction or elimination of the precancer cells. Table 1 shows the list of antibiotics and hormones that have been tested against the precancer cell complex. Unfortunately, no agent has been found that will provide a universally favorable effect, although random cell response has been observed when the following agents have been used. In most cases, the response is transient. In a series of cases however, in which the crude Aureomycin suppositories were used, complete and permanent regression of the precancerous

TABLE 1

RESEARCH CYTOLOGY: COMPOUNDS SCREENED IN PRECANCEROUS LESIONS OF CERVIX

Antibiotics

Penicillin	Usnic Acid[‡]
yellow crude	Sodium Usnate
topical	Polymixin[§]
Aureomycin[*]	Thiolutin[§]
crude suppository	Magnomycin[§]
refined suppository	Sarkomycin[‖]
Streptomycin	Bacitracin[§]
Terramycin[†]	

[*] Provided by Lederle Laboratories.
[†] Provided by Pfizer & Co.
[‡] Developed by Dr. Alfred Marshak, Woods Hole, Mass.
[§] Provided by Pfizer & Co.
[‖] Obtained through the Public Health Foundation for Cancer and Blood Pressure Research, Inc.

TABLE 2

PRECANCER CELL PROGRESSION STUDY

Time interval: ½ to 6 years
No biopsies
Cases: 339
Progression: 121 cases
Final tissue diagnosis:
  92 Carcinoma *in situ*
  29 Early infiltration

TABLE 3

RANDOM CELL RESPONSE

Aureomycin (crude)	Sarkomycin cortisone[*]
5,6-Dimethylbenzotriazole	(Thiolutin) Magnomycin

[*] Supplied by Merck & Co.

TABLE 4

RESEARCH CYTOLOGY: COMPOUNDS SCREENED IN PRECANCEROUS LESIONS OF CERVIX

Hormones

Estron	Stilbesterol
Progesterone	Testosterone
Androsterone	Cortisone
ACTH	

lesions resulted. It should be emphasized that this is not consistent an
that there seems to be some factor of individual predisposition since, in th
cases showing favorable response, there is an early allergic reaction involving th
cervical tissues.

Glycogen

It is possible to do sensitive glycogen cervical cell studies using the modifie
Best carmine-staining techinque. Clumps of glycogen are clearly eviden
throughout the cytoplasm of normal cells. On the other hand, malignant an
premalignant cells show a complete absence of glycogen. Serial studies c
premalignant lesions may enable the recognition of experimental hormona
agents that cause glycogenesis, which may precede morphological response
We have not yet succeeded in finding such a hormone or antibiotic. Th
search, however, would appear to be worth while. Reports of other profit
able cytochemical studies employing the exfoliative cell techniques are mad
elsewhere.

Tissue Culture Studies

C. G. Grand, working in our laboratory, has made intensive investigation
of the behavior of premalignant cells in the human cervix in tissue cultur
He has studied the variable velocity of outgrowths from explants. It woul
appear that the growth velocity in these cell types is midway between the i
flammatory and the malignant, and the character of growth resembles that
actual carcinoma *in situ*. Efforts to influence the precancer cells growing i
tissue culture with hormones such as estrogen and cortisone, applied to th
culture media, have failed to reveal evidence of growth influence under thes
conditions.

Defense Mechanisms

There has long been uncertain evidence that some tumors, even when we
established, will undergo spontaneous regression. But scientific evidence ha
been difficult to procure. Ewing, in his treatise on neoplastic diseases, assume
that there existed, within the body, defensive reaction against maligna
growths. It may well be that preinvasive cancers may remain latent for yea
because of such body resistance. Numerous authorities have expressed th
belief that inflammation around tumor masses represents the principal defe
sive measure. Possibly, with age, the "inflammatory potential" of the tissue
diminishes, thus releasing the tumor cells from restraint, causing invasio
infiltration, and metastasis to follow.

Stewart has reported new cases of "spontaneous regression" of neoplast
disease in man. Direct damage to cancerous tissue has been induced wit
subsequent resorption of necrotic parts accompanied by severe systemic rea
tions, followed by regression of the neoplasms.

Gellhorn summarized the problem of the natural defenses against cancer a
follows: "The evidence for inherent defense mechanisms against human cance
is tenuous and yet, on occasion, very persuasive. Thus, the rare but wel
documented cases of disseminated tumors which spontaneously regress or th

less infrequent instances of recurrent cancer many years after removal of the primary tumor suggest that there may be physiological mechanisms which can either destroy malignant cells or hold them in check for long periods."

It would appear that the experimental production of an "alarm" reaction, such as an induced acute inflammation, may stimulate the body's resistance to cancer. Four years ago, we reported regression of carcinoma *in situ* of the cervix in a minority group of patients receiving Aureomycin by topical vaginal suppositories, but later studies showed that the effect was inconsistent. More recent experiments have shown a similar regression in occasional patients induced by two other agents, 5,6-dimenthylbenzotriazole and a combination of thiolutin and Magnamycin.

Now, in retrospect, we realize that those patients showing maximal regression were those in whom hyperemia of the cervix with exudation of fluid was noted. Yet, despite moderate pruritis, the patients were cooperative and treatment was continued. In other words, we were creating an alarm reaction, an acute local inflammatory process. We now speculate whether the specific character of the chemical agent or antibiotic had anything to do with the end result and whether the nonspecific inflammatory reaction, linked with individual predisposition, may have been responsible for the complete and permanent regression noted in some cases of carcinoma *in situ* and for the partial or temporary regression noted in the majority. These investigations are being continued.

Conclusions

(1) The life history of cancer of the cervix may be fully explored with this splendid new tool. Its duration: extremely variable, up to 20 years. Probably all cervical cancers pass through a premalignant stage, during which cells of a precancerous type may be identified. The velocity of growth varies within wide limits. We estimate that in perhaps 10 per cent of cases, velocity of growth is such that the premalignant stage is passed through within a year or less; in about two thirds of cases, within one to five years; in 23 per cent, perhaps more than five years. We have observed progress from normal cells to early infiltration in one case during an 11-month period. At the other extreme, we have observed a premalignant lesion that remained relatively static during an eight-year period of cell observation.

(2) A false picture of the life history is portrayed following simple or multiple biopsy, since apparent regression was noted in 18 lesions and following cautery in 22 lesions. On the other hand, cautery failed to effect regression in 51 lesions.

(3) Progression from a premalignant to a malignant-stage carcinoma *in situ* was observed in 92 cases studied during a period of six months to six years preceding confirmatory biopsy.

(4) Progression from premalignant grade II to malignant grade III, B or C, with tissue confirmed as showing invasive cancer during a six-month to six-year period, 29 cases.

In nine cases, it has been possible to link up the normal cell stage with carcinoma *in situ* six months to five years later while, in one case, progression from normalcy to early infiltration was observed.

CELLULAR CHANGES ASSOCIATED WITH INFLAMMATION

By Elliott D. Blumenthal and E. L. Hecht
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The cytologic picture in patients with inflammatory conditions of the genita tract may be confused, at times, with malignancy. It is the purpose of thi paper, therefore, to evaluate the confusing cellular patterns associated with inflammation.

When inflammation is present, clumps of leucocytes often obscure cellula detail. Two types of cells are generally seen: several varieties of histiocytes and atypical cervical cells. Chronic inflammatory lesions of the cervix are by far the most frequent cause of cell atypicalism. Smears from these cervices consist of sheets of leukocytes, histiocytes, bacteria, and blood. The cells may be markedly enlarged, but the nucleocytoplasmic ratio remains unchanged In many instances, the cytoplasm is not evident. In addition, various types of atypical epithelial cells appear. The parabasal cells from the ectocervi may appear quite bizarre, with large vacuoles and irregular cytoplasmic processes (FIGURE 1). In cases of endocervicitis, the endocervical cells show variation in size, and the cytoplasm is not evident, leaving free nuclei that may appear malignant (FIGURE 2). The nucleochromatin, however, remains homogeneous. Infection frequently causes both the cytoplasm and nucleus to stai more deeply, giving an impression of hyperchromatism. Thus, smears from severe chronic cervicitis may show certain cellular characteristics not unlik those of malignancy; namely, large nuclei, irregularity in size and shape o nuclei, and some degree of hyperchromatism.

The cytologic picture in erosion of the cervix is practically identical wit that of chronic cervicitis. The nuclei of these cervical cells are similarly enlarged, and show some variation in size, shape, and degree of hyperchromatism

Squamous metaplasia or epidermidization of the cervix is a benign conditio that is always accompanied by an inflammatory process. This entity may b recognized cytologically by the profuse numbers of endocervical cells that ar present on the smear. These cells show an even greater degree of atypism with more pronounced variation in size, shape, and staining reaction of th nuclei than is evident in chronic cervicitis, or chronic cervicitis with endocervicitis.

In various inflammations of the vagina, similar cellular changes are present Senile vaginitis is a common infection in which there are abundant leucocytes histiocytes, and outer basal layer cells, many showing variation in size, enlargement of the nuclei, and hyperchromatism (FIGURE 3). Hecht,[3] in a stud of 378 menopausal patients, found on 3.16 per cent of all the smears an abnormal pattern similar to that seen in carcinoma; namely, vacuolization of cytoplasm, nuclei that are bizarre in shape, hyperchromatic, relatively large, an eccentric. Two of these cases developed cancer of the cervix two and fiv years after the initial study. This would indicate that when *marked* atypica

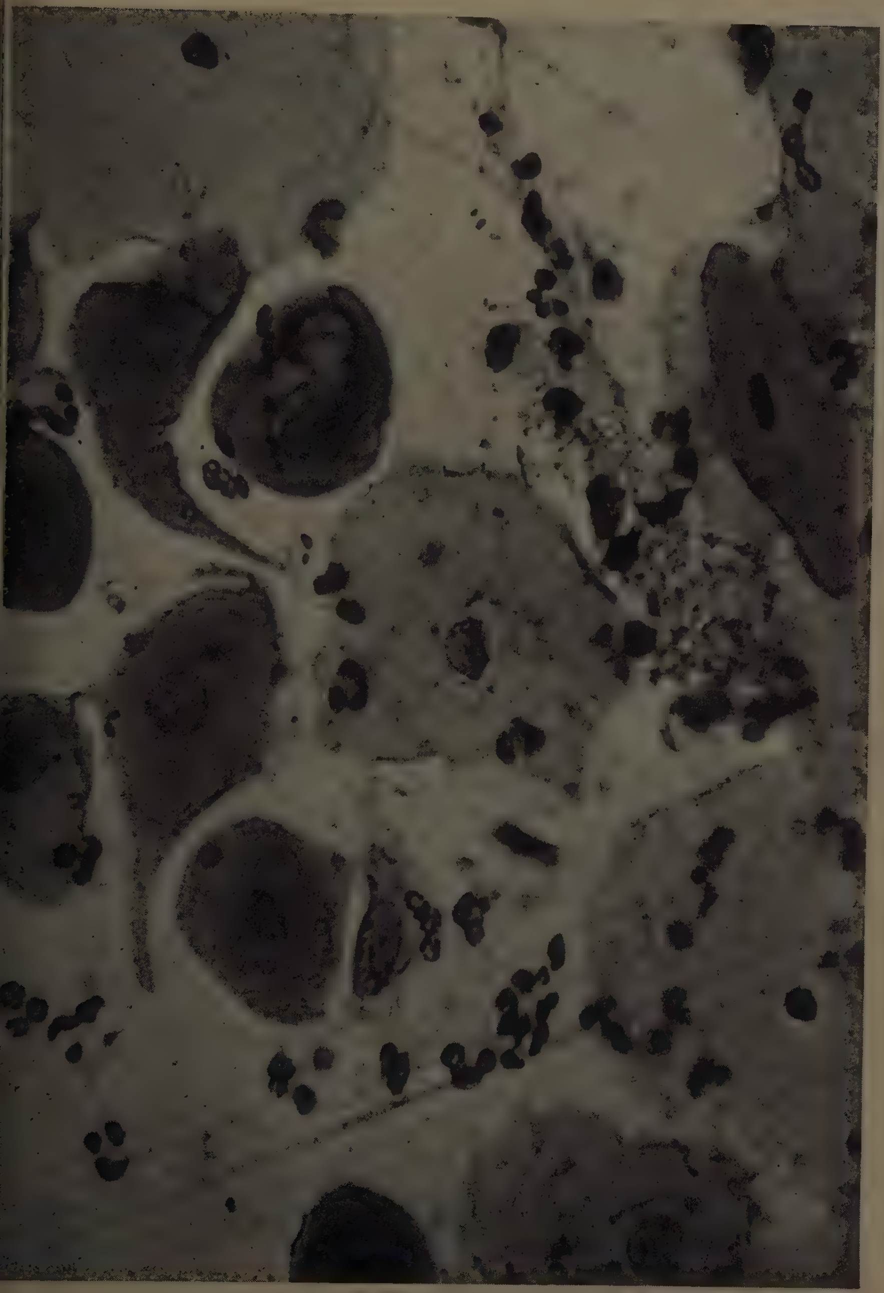

FIGURE 1. Cervical irritation cells showing irregularity in size and shape of nuclei with some degree of yperchromatism.

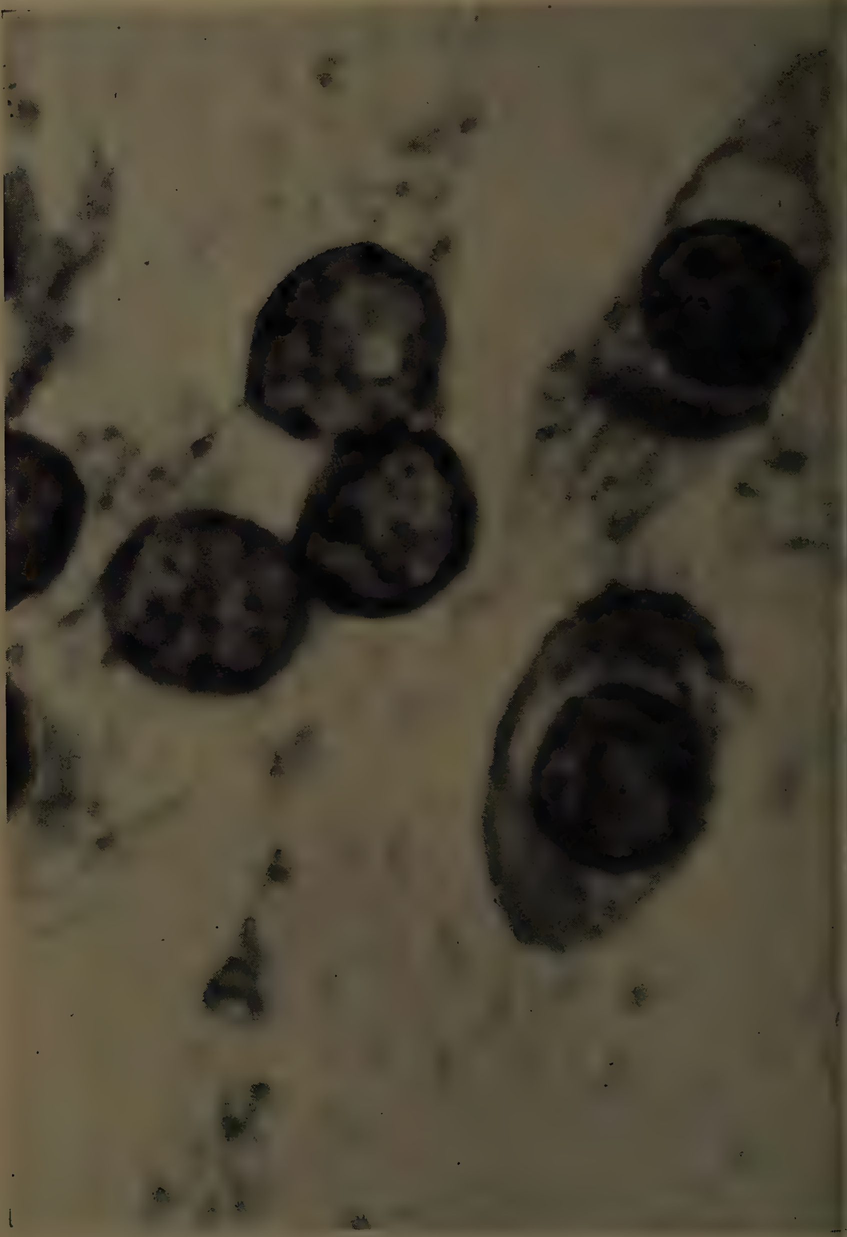

FIGURE 2. Endocervical cells showing hyperchromatism, from a case of chronic endocervicitis.

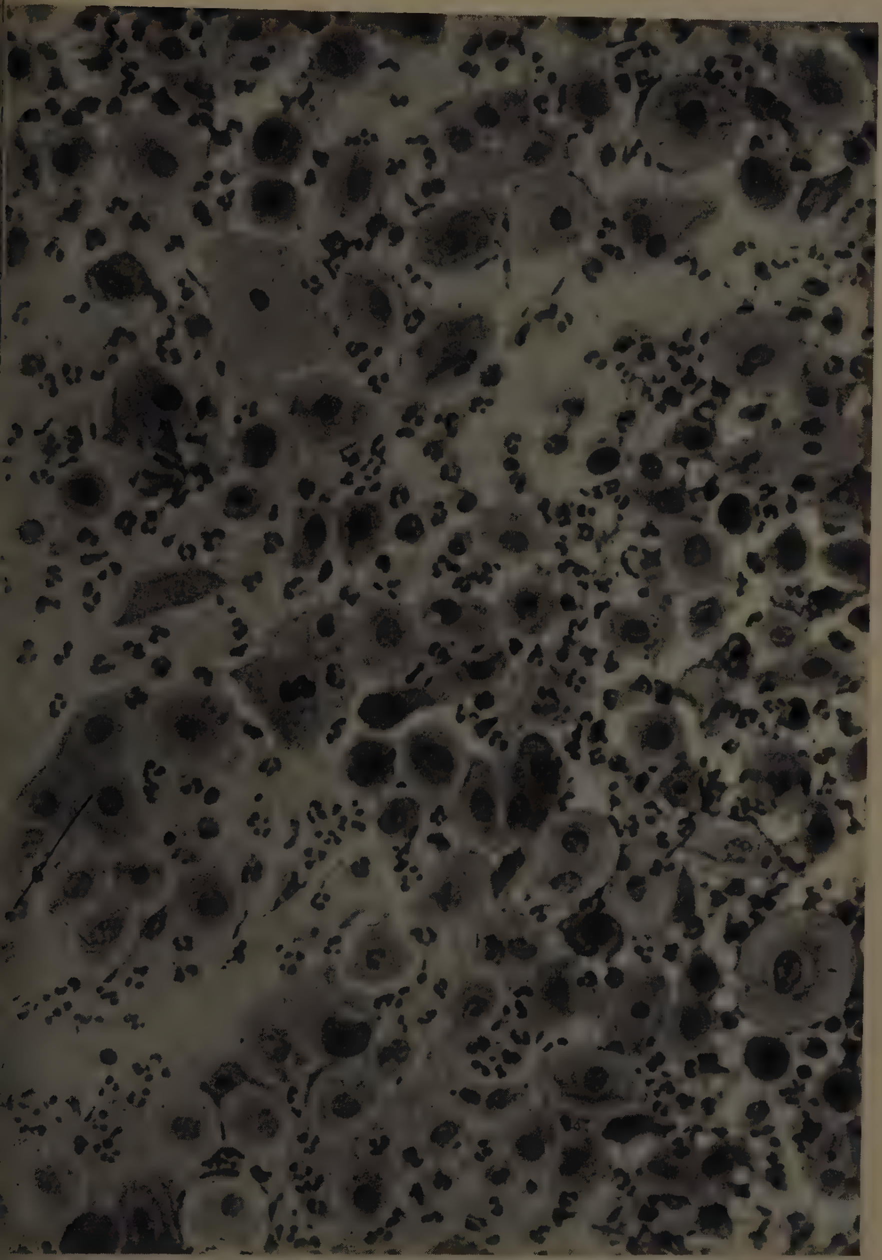

FIGURE 3. Atrophic menopausal type smear showing variation in size, shape, and staining reaction.

cellular material is found, patients must be followed closely, since the proce may be irreversible.

Trichomonas vaginalis and *Monilia albicans* are two parasites that frequent infest the vagina and produce chronic inflammatory changes. They are easi recognized cytologically. The trichomonads may be identified as oval, greyis structures, with small, reddish elliptical nuclei near one pole. The flagel sometimes may be seen in well-preserved parasites. The monilia spores ma be recognized as small elliptical structures that take a faint eosinophilic stai These parasites are often associated with the cellular atypism previously d scribed. They cause changes in the precornified and outer basal layer cell often producing false cornification, degeneration of the cytoplasmic border and hyperchromatic nuclei.

In endometritis, there is also an influx of leucocytes and histiocytes. Atyp of the endometrial cells in this entity occurs just as in other inflammator conditions of the genital tract, and the presence of large numbers of histi cytes resembling adenocarcinoma cells (FIGURE 4) may be cytologically co fusing.

One of the most commonly misinterpreted of all cells is the histiocyte whi is seen in infection (FIGURE 5). Histiocytes vary in form and size; the sma histiocytes have oval, round, or bean-shaped nuclei with finely vacuolate cytoplasm and without a well-defined cytoplasmic border. The large histi cytes have large vacuoles containing phagocytosed material. The third ty is the giant cell with several nuclei arranged peripherally in a foamy cytoplas (FIGURE 6). Histiocytes, to the inexperienced cytologist, may simulate oth types of cells. The nucleus of the histiocyte, however, usually presents vesicular pattern. There is no irregularity of the chromatin network, nor hyperchromatism evident. Further, the nucleochromatin is always even and finely distributed throughout the entire nucleus.

In the past three years at the Cytology Laboratory of the University Hosp tal, New York University-Bellevue Medical Center, routine vaginal and ce vical smears from 15,248 women have been examined. All cases diagnosed "positive for malignancy," approximately 0.5 per cent, have been eliminate from this discussion. Of the 15,248 cases, 13,265, or 85 per cent, showe varying degrees of chronic cervicitis by the cytologic method. Cellular atyp was severe enough to warrant the diagnosis of "suspicious but negative" 178 cases. Of these 178, biopsies were obtained in 145, and the pathologic diagnoses were as follows: severe chronic cervicitis and endocervicitis in 7 squamous metaplasia and endocervicitis in 45; basal cell hyperplasia in 1 intraepithelial carcinoma in 3; and invasive carcinoma in 2.

All of these cases, except the five diagnosed as carcinoma, were then treate with cautery and followed at intervals by means of the vaginal and cervic smears. Generally, as the lesions of the cervix healed, the atypical cells tende to disappear from the smears. The atypia associated with inflammation thus apparently reversible.

Inflammation, then, produces atypia in the benign cell, but rarely is a

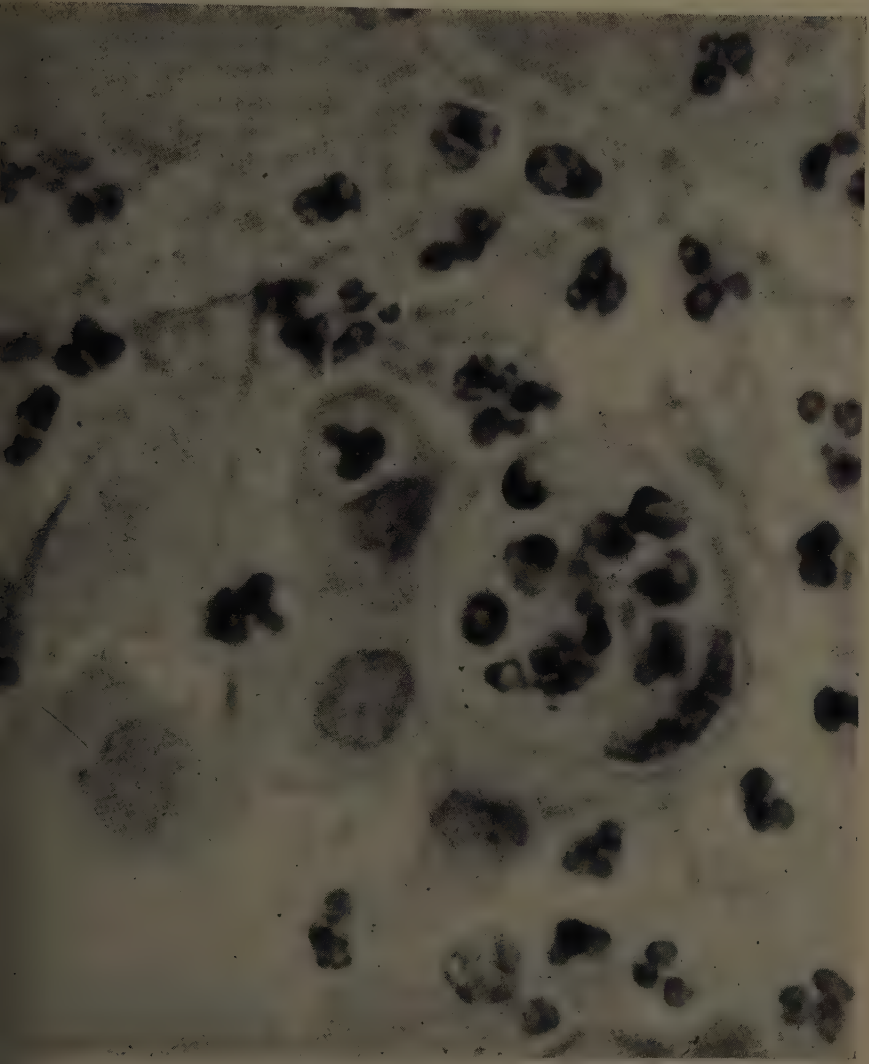

FIGURE 4. Large histiocyte containing phagocytosed leukocytes; this may be confused with adenocarcinoma ll.

ciated with the severe changes found in malignant nuclei, such as clumping the chromatin, marked irregularity in the chromatin network, and severe teration of the nucleocytoplasmic ratio. It becomes apparent from these servations that a diagnosis of malignancy is not based upon a single criterion, it on a total picture that includes many factors.

The diagnosis of "suspicious but negative" was made early in the present udy. Increased experience has made such a diagnosis rather rare in cases of

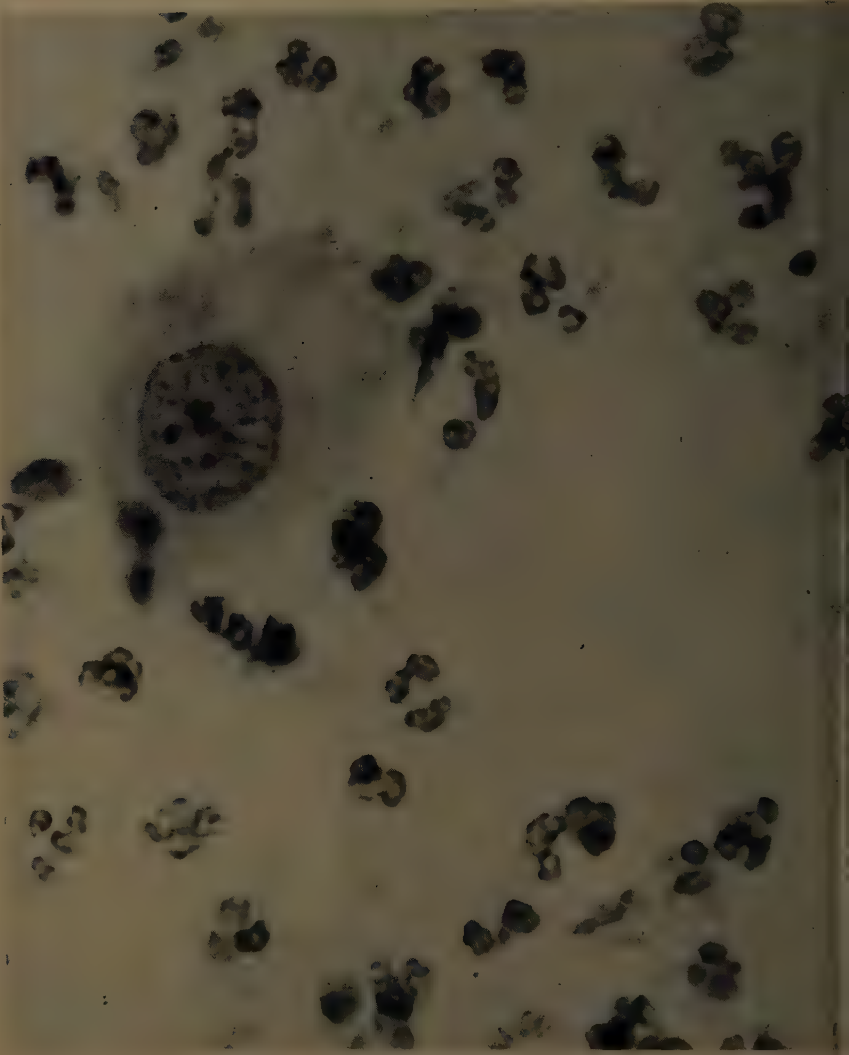

FIGURE 5. Histiocyte with large vesicular nucleus.

cervical pathology. The milder degrees of cellular atypism present in infla
matory conditions are seldom, at the present time, confused with malignan

Because of the presence of atypical cells in genital tract infections, so
investigators have postulated that chronic inflammation may be a predispos
factor in the pathogenesis of cancer of the cervix.[2, 6]

Ayre[1] studied cervical biopsies showing abnormal growth proliferation in
presence of cervicitis, low thiamine excretion, and excess tissue estrogens. I

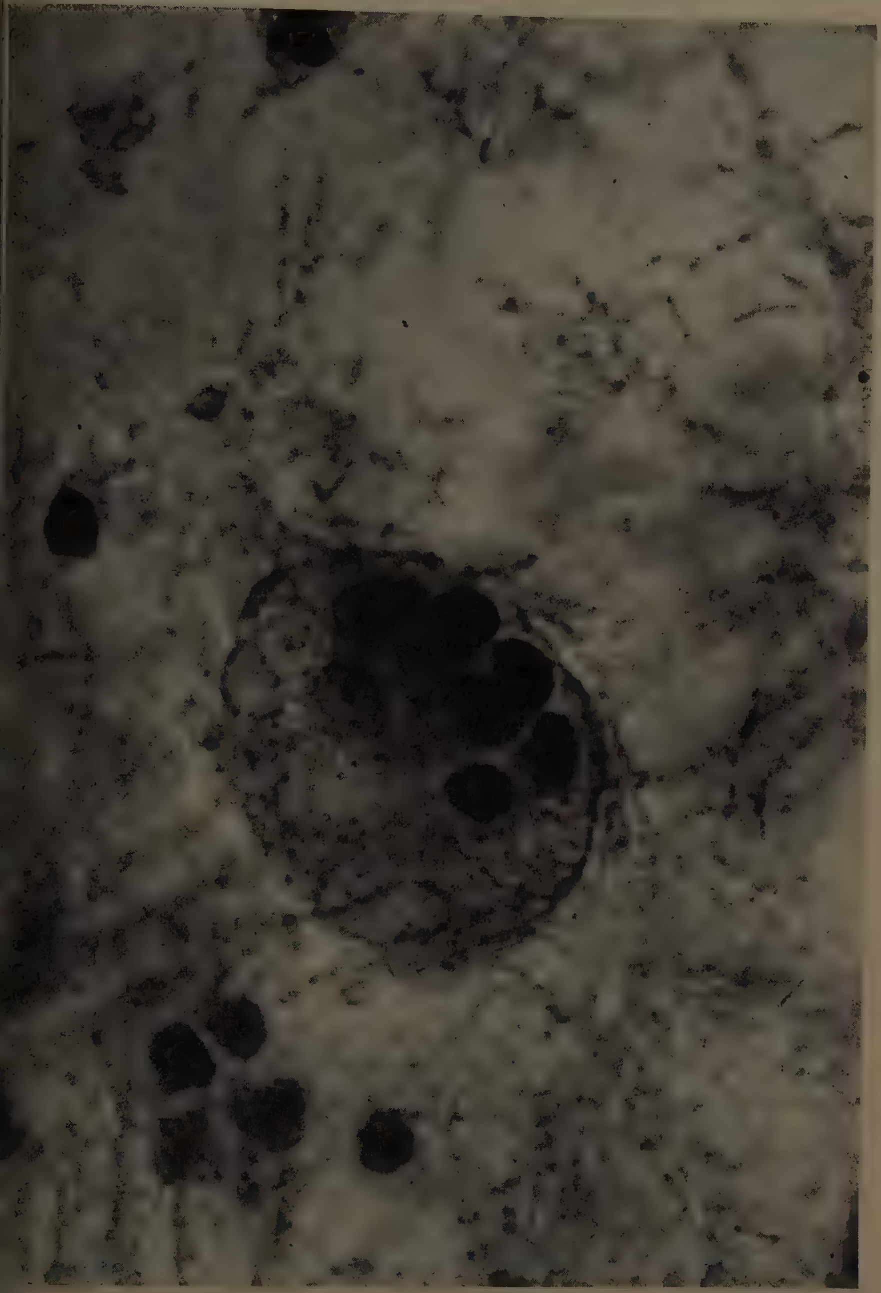

Figure 6. Giant histiocyte with multiple nuclei.

excess tissue estrogen findings were based on comparative vaginal and cerv cornification counts, a method that may be open to question in the presenc inflammation. His work was an attempt to correlate infection, excessive tis estrogen, and nutritional deficiency in the production of squamous carcin of the cervix. In the present study, excessive cornification has been m frequently seen in the presence of infection, and is in no way related to development of cancer.

Saltzstein and Topcik[8] found that, of 18,562 patients who were conside adequately treated for cervicitis, only 15 later developed cancer. Of 2 patients with known cervical cancer, however, the authors reported that c 33 had had adequate treatment of pre-existing cervical lesions.

It is certainly possible that chronic irritation is a factor in the developm of cancer elsewhere in the body. But, what about cancer of the cervix? A has suggested that the so-called hyperactive inflammatory cells seem to rep sent a morphologic stepping stone between the normal cell and the cell descri as a precancer cell. This paper has indicated that chronic inflammation the cervix may produce on the smear a marked cellular atypia at times diffi to distinguish from malignancy. Such atypia, however, rarely satisfies over-all criteria for malignancy and, to the experienced cytologist, does now present a serious problem.

A number of recent papers[4, 6] show that, over a period of years, benign b cell hyperplasia of the cervix may progress to intraepithelial carcinoma then on to invasive carcinoma. On the other hand, chronic cervicitis is extremely common entity, occurring in 85 to 90 per cent of the routine sm examined at the University Hospital. But cervical carcinoma is rather r In some of the most severely eroded and irritated cervices, thorough pat logical examinations have failed to reveal carcinoma; and there is no do that cancer develops in cervices in which no irritation is present. Koss Durfee[5] have followed cytologically 8 patients in whom intraepithelial cinoma developed from 12 to 71 months after the period of observation be They state that the cytological changes are essentially insignificant in nat and cannot be established as characteristic precursors of the malignant char eventually seen on the smears.

To summarize: (1) inflammatory lesions of the genital tract may prod cytological atypicalism, rarely satisfying, however, all the criteria for ma nancy; (2) mild cervical cell atypism associated with inflammation is a versible condition, disappearing after treatment of the infection.

It may be concluded that there are probably many factors involved in production of cancer of the cervix, and that, though chronic inflammation m be one of these factors, it is one of the less important. It is the only kn factor, however, which can be eradicated by treatment.

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PRECANCER CELLS IN AMPHIBIAN ADENOCARCINOMA*

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For many experimental biologists the problem of precancer is gaining incre ing significance. If one attempts to answer the question whether or not th is such a thing as a precancer cell, it is first necessary to have experimen evidence. There must be no room for doubt as to whether the changes are m tiple, gradual cell transformations or, by contrast, are derived from single-ev abrupt somatic mutations. In considering the etiology of this disease, it often difficult to separate the factors contributing to the pathogenesis of first cell to become malignant from a presumably different set of factors th result in a tumor. The concept that cancer arises from a single cell ev leading to a progeny of neoplastic cells has been widely held. In speaking human cancer Paul Steiner (1954) says that "once carcinogenesis has pass an irreversible point, a tumor is inevitable." In another place, he states th "after a cell has been changed from normal to neoplastic, its further multi cation to form a new protoplasmic mass, called a tumor, is under the cont of a set of factors different from those that initiated the tumor." There another point of view, however, as experiments with the cells of the amphib kidney both *in vivo* and *in vitro* show; namely, that the etiologic agent for re adenocarcinoma will pass through a fine porcelain filter and that not one many cells are affected concurrently by the filtrate. In the experiments ported in this paper, over one third of the injected frogs showed abnormalit of the kidney tubules, including transformations of pre-existing cells, and ferent degrees of conversion into acinarlike structures that, when taken gether, can be interpreted as stages of precancer. Approximately one fi reached the point of microscopically identifiable adenocarcinoma, but the mainder were negative and presumably immune. Thus, while the prepond ance of the evidence here favors the orthodox method of tumor growth by tis cellular *invasion*, there are clear indications that the tumor may also spread *infection*; *i.e.*, by a slow-acting, "organ-specific" virus, as postulated by Lu (1952).

Adenocarcinoma of the kidney of the leopard frog (*Rana pipiens*) off unusual advantages over mouse or other mammalian neoplasms for the stu of malignancy at the subcellular, cellular, organ, and animal levels. Not o are the cells hardier and among the largest cancer ones known (averag 150 × 50 μ in tissue culture, with multinuclear extremes up to 600 × 350 but they *uniquely* combine, as Lucké showed, a presumable virus origin wit specific type of intranuclear inclusion body. With an electric micromanipu tor, it is possible to isolate fresh unfixed cell components, such as nucle chromosomes, and inclusion bodies. We have also found that excised pie

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[*] The research described in this paper was carried on while the author was a member of the staff of the Nati Cancer Institute, Bethesda, Md., between 1950 and 1955, while he was assigned to the Biophysics Section of Carnegie Institution's Department of Terrestrial Magnetism. Grateful acknowledgment for the support facilities provided is hereby made.

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of tumor can survive 6° C. for 10 to 12 weeks, thus providing standard material for culture and experimentation. In extension of the excellent work of Lucké into new areas, results reported in this paper show that cell-free aqueous extracts of frog tumor brei, obtained after high-speed centrifugation and passage through an 02 Selas porcelain filter (maximum pore size 0.85 μ) will produce histologically differentiated adenocarcinoma in as little as seven weeks, if injected directly into the kidney. Using improved tissue culture methods and liquid media, Duryee and Doherty (1954) found that nuclei of tumor cells are the major source of the cytoplasmic inclusion bodies, and that nuclei of both normal and neoplastic cells function chiefly as secretory organoids. Attention is directed to new data here on hyperactivity of the neoplastic nucleolus, together with characteristic diffuse DNA, or possibly pleoform DNA coacervate droplets, as contrasted with the little or no DNA in nucleolar cores of normal cells both *in vitro* and *in vivo*.

Once the existence of a filterable agent was established, it was possible to go back to sections of spontaneous tumors of various sizes and degrees of malignancy (*cf.* Lucké, 1938, "spectrum of malignancy") to restudy the microscopic areas just beyond the nodular walls and in the residual tabs of normal kidney tissue on the large tumors and in the opposite kidney from a unilateral tumor. In 15 out of 21 series of serial sections, there was some degree of precancerous change; *i.e.*, evidence of transformation of normal tubules toward the acinar pattern. It is to be emphasized that the term "precancerous" is presumptive only so far as it applies to the possible future malignancy of any tissue taken out for examination. Contrary to wide belief, however, the term is amenable to new and useful definition, and the criteria of precancer may now be examined experimentally in tissue cultures by application of exogenous agents to normal cells. For this paper, the following generally reliable but not perfect criteria for precancer are defined in sequence of importance: (1) hyperactivity of the nucleolar colloid based on abnormal size and abnormal DNA content; (2) hypersecretion of nucleolar organized colloid through the nuclear membrane into the cytoplasm to form transient inclusion bodies; (3) change in cell form and increased basophilia of the cytoplasm; (4) mitosis in the tubule wall and appearance of supernumerary layers of cells; and (5) new growth characteristics in the animal and in culture, with special reference to increase n amount of nuclear secretion.

Perhaps the most instructive examples of precancer were found in the residual tabs of "normal" kidney left on some large and rapidly growing spontaneous adenocarcinomas weighing six grams or more. Sections through these areas illustrate graphically the *last stages of normality*, even better than some microscopic tumors illustrate the *first stages of malignancy*. In both cases, he remaining functioning tubules gave signs of reacting defensively a sort f infectious process, comparable in some regards with phage infection in *Escherichia coli*. Also instructive were the different degrees of malignancy hown. These could be distinguished by the extent of metastastis or, where his was lacking, by the diffuse character of the growth throughout the kidney, n contrast to isolated and restricted nodules. Form and character of cellular

differentiation toward acini were found useful criteria. Rate of growth a
was used as a measure of hyperplasia rather than degree of malignancy.

As further background for this problem and in support and in consiste
with the present interpretations are the extensive and valuable statist
analyses of Lucké on the incidence of spontaneous tumors of Vermont f
obtained from the same dealer* as ours. It is pertinent to the precar
aspects of the disease to recall from Lucké's review (1952) that multiple add
carcinomas were found in 716 of 1429 tumor frogs that he examined, and
involvement of both kidneys was discovered in 63 per cent of the total num
Thus, the facts of multiple foci and bilateral occurrence in the large majo
make very difficult any insistence that carcinogenesis in these amphibi
merely a single cell event such as a somatic mutation, but they do indi
quite the opposite type of event, namely gradual and wide-spread pathogen
In two extreme cases, due apparently to high potency of the agent and/or
resistance of the host, large spontaneous bilateral tumors have been rece
observed (*e.g.* FIGURE 1) in the form, proportions, and vessel relationship
normal kidneys, but weighing as much as three grams each for right and
respectively. These *bilateral replica tumors*, together with the gradual
cancerous changes seen in the filtrate-injected frogs, and the alterations of
maining kidney tabs on large spontaneous tumors offer clear and cogent
dence that, for these animals, carcinogenesis is not confined to a single
nor is it an all-or-none phenomenon. They all agree in showing that
cinogenesis is a concurrent series of many infectionlike events.

The hypothesis here is offered that amphibian renal adenocarcinoma is
marily a virus infection of the nucleoli of proximal tubule cells. Implicat
of these data for a general theory of carcinogenesis will be found in the
cussion.

Materials and Methods

Observations were made on Lake Champlain frogs obtained from dealer
Alburg, Vt. The spontaneous tumors reported in this series arose in 41
mals, of which 34 were males. Eighteen of the total were bilateral in ori
and constitute 44 per cent. The largest tumor mass found at autopsy fro
female weighed 14.1 gm. and was bilateral in character. The smallest tum
recognizable grossly were approximately 1 mm. in diameter. Of interes
the finding of 2 spontaneous bilateral tumors in a series of 21 animals ship
as "normals" and kept in the laboratory 4.5 months. Tumor diameter a
aged 10 mm. in each kidney, thus indicating the minimal rate of growth f
spontaneous neoplasm.

In order to follow carcinogenesis *in vivo* over the long periods necessary a
at the same time, to observe cytological changes *in vivo* due to exactly the sa
filterable agent, the following over-all experimental plan was instituted. T
years of preliminary experimentation were necessary to standardize the te

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* Natural incidence of frog tumors is not precisely known. The dealer (C. H. Mumley of Alburg, Vt.)
supplied Doctor Lucké, claims that he sent extra tumor frogs in boxes to Philadelphia, so that Lucke's est
of from 3 to 6 per cent is high. The dealer stated it was necessary to palpate 35,700 frogs to find 19 tumor
for the author in 1949–1950. On the other hand, he described certain swampy tributaries of Lake Cham
highly contaminated with sewage, where tumor bearing frogs might be occasionally found.

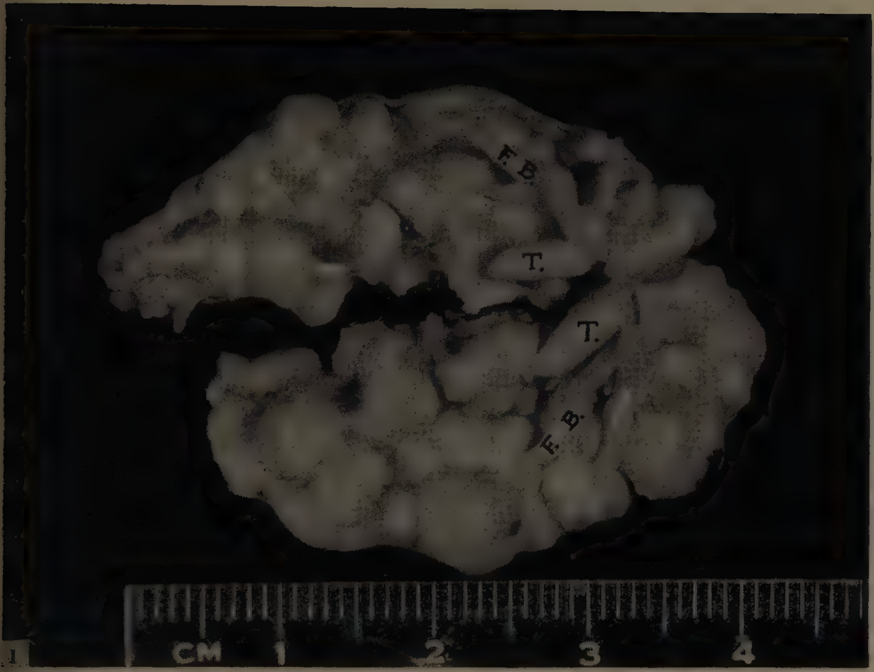

PLATE I

FIGURE 1. *Spontaneous bilateral replica tumor.* Both kidneys of male *Rana pipiens* completely transformed into adenocarcinoma. Note attached testes (T.) and fat bodies (F.B.). Animal developed sudden massive edema three days before death. Replica format indicates tumorgenesis must have developed concurrently from many foci.

niques and to show the essentiality of interlocking information derived fr simultaneous studies conducted at the virus level, at the cellular level in c tures and in the kidney, and at the organ or animal physiology level. F techniques, see Duryee and Doherty (1954) and V. Bush, Duryee, and Hasti (1953). The type of procedure, as finally adopted, was as follows.

First, a filtrate from a spontaneous tumor of medium malignancy weighi 5.46 gm. was prepared (October 26, 1953), divided into eight aliquots of 0.1 each, and injected into the left kidney of eight medium-size male frogs (ser 6, TABLE 1). A fragment of tumor tissue was fixed in Tellyesniczky's flui for sections. Also, a small piece was cut into approximately 1 mm. cubes starting 18 lines in tissue culture. Sublines of these cells (series FT-21) we carried 53 days with sample cultures fixed and stained at weekly interva Living cells in culture were observed every two days under phase-contra lenses and were photographed when details of unusual interest were seen. this way, base lines of comparison were established for all future experime during the next year. In three cases, tissue cultures of Vermont tumor mater were transferred to the anterior chamber of eyes of other frogs, where one ga rise to renal adenocarcinoma.

Preparation of tumor filtrate. Large tumors were easily palpated throu the body wall. The frogs were pithed, and the bodies rinsed and swabb with 70 per cent alcohol. The body wall of each was opened with ster instruments and reflected, so that the tumor could be dissected free witho touching the skin. Tumors were transferred to sterile aluminum foil for ra weighing, then cut into pieces and placed in an Elvehjem blendor and grou in sterile distilled water, 1 ml. per gm. wet weight. The resultant suspens was poured into a 10-ml. sterile glass vial with pyrex glass beads and mecha cally shaken in a magnetic vibrating shaker for 10 minutes. Supernate liq and foam was pipetted off into centrifuge tubes. At this stage, microsco examination showed only a suspension of fine granules and droplets and f intact nuclei. Very rarely, an intact cell could be found. It was conclud that the magnetic vibrator broke at least 99 per cent of the cells. In th experiments, the suspension was diluted with an equal volume of double py distilled water and centrifuged at approximately 2000 rpm. for 10 minut In others, phosphate-buffered (pH 7.1) 0.1 M NaCl was used. Under pha contrast lenses, the supernate contained only numerous granules in act Brownian motion and refractile clear vesicles varying from 0.5 to 1.5 μ. the next centrifuging of the supernate showed no pellet, it was ready for filt tion; otherwise, a third centrifuging was carried out as before. Usually, 3 5 ml. of clear pinkish supernate was obtained, having a pH of 7.3 as measu with a Beckman glass electrode. Smears stained with Feulgen reagent show small clumps of black and red granules approximately 0.2 to 1.0 μ. The cl supernate was then put through an 02 Selas filter fused into a porcelain fun cleaned and sterilized, with a suction-flask holder and 10 ml. test tube in pl inside. Maximum pore size is rated by the manufacturer as 0.85 μ. Af 10 to 15 minutes of filtration, using a water-tap aspirator for vacuum, appro mately 1 to 2 ml. of clear pinkish or slightly amber filtrate was obtained. Under 44× phase-contrast objectives, only very few granules 0.5 to 0.8 μ could be found per field. Feulgen-stained smears were not practical. The filtrate was drawn up in a graduated syringe with a No. 25 needle, and 0.1 ml. portions were injected into each host.

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* Tellvesniczky's fluid (Fekete's modification): 70 per cent ethyl alcohol, 20 parts; formalin, 2 parts; gla acetic acid, 1 part.

Injections into normal frogs were made in the following manner after tests with sagittally-sectioned lumbar areas taught the proper angle for most effective kidney punctures. Multiple kidney punctures were accomplished by oblique insertion designed to avoid injury to the lumbar plexus and subcardinal sinuses. Most punctures were made unilaterally, but no uniform correlation with the site of induction was found. For most series, controls were made by injecting two animals similarly, each with 0.1 ml. sterile NaCl. Kidneys of all controls were negative.

It may be recalled that Lucké reported that numbers of induced neoplasms with frozen pulverized and glycerinated tumor preparations increased after three months. Our low-temperature survival experiments throw some doubt on his use of this type of test for proof of virus action. Our series included only four frogs kept over three months, inasmuch as it seemed desirable to search for the earliest signs of change and the possibility of definite forms of precancer. The question of feeding and maintenance of injected frogs is complex. It was decided to change the early procedure of keeping the animals without feeding, at room temperatures, since adquate nutrition might increase the rate of possible tumor growth. Consequently, the last five series were fed weekly with 2 to 5 gm. chopped raw beef liver, because this had been found to be a complete diet for all species of salamanders. Later, frogs were kept in constant temperature boxes at 15° C. and seemed to live well and with sharply lower infection rates.

Routine fixation of kidney and other organs of interest with lesions were carried out with Bouin's or Tellyesniczky's fluids. To reduce the onerous task of searching complete serial sections of both kidneys it was suggested by the Tissue Department of the National Cancer Institute to take alternate two-inch segments of paraffin ribbons of kidneys cut longitudinally. Even with this improvement and with thick sections (10 μ), the total number of slides of serially sectioned material numbered approximately 1800. In many series, sample slides were stained with Giemsa and others with Feulgen, and the majority with standard hematoxylin and eosin. For special microphotography, some segments of blocks were cut at 4 μ. Due to the over-all plan of interlocking experiments, it was possible to have sections of the donor tumors of both "high" and "low" malignancy to compare with their respective induced ones and with the live tissue culture and stained cells of homologous strains.

Tissue culture methods. Since a major purpose of this work was to investigate the physiological role of the nucleus in carcinogenesis, methods of tissue culture had to be improved and modified. It was necessary to eliminate the standard type of plasma clot for micromanipulation and to devise moist chambers and tools suitable for use with long-working distance phase-contrast condensers. Small squares of Earle's perforated cellophane held the explants

in hanging drops in Romicron slides. Grateful acknowledgement is made
Doctors V. Bush and M. A. Tuve, and to the technical staff of the Carneg
Institution of Washington, D. C., for help in devising an electric micromanip
lator for isolating nucleoli, inclusion bodies, and other components of norm
and malignant cells in culture. With new ultraprecise micropipettes no
under development, it is hoped to transfer these components from cell to ce
to avoid, as far as possible, the present objections and difficulties that atta
to all kinds of cell macerations.

With explants of tumor or normal kidney in chick embryo extract and hor
serum media, heavy growths always were obtained. The acinar patterns
the tumor resolved into monocellular sheets with identifiable individual mali
nant cells, thus facilitating photographic recording of cytological change
Cultures withstood 30-minute illumination from a 250-volt ribbon filame
bulb at 23 inches from the condenser, provided a green Wratten No. 58 filt
was used. A specially designed Zeiss Miflex camera was mounted vertical
on a Leitz triocular unit with movable prism, so that photographs could
made with minimum vibration and negligible interference with visual observ
tion.

Twenty-four series of adenocarcinoma cultures were carried on in sequenc
concurrent with 10 series in sequence from normal kidneys. Each series co
sisted of from 8 to 20 parallel cultures made from a single donor tissue.
mentioned earlier, it was found that excised tumor tissue grew vigorously aft
long periods at near-freezing temperatures. Although whole pieces survive
longer if the medium was renewed at weekly intervals, nevertheless moi
fragments 1 cm. in diameter survived 27 days without any media and, late
yielded cells that grew actively in culture. Occasionally whole cultures we
simply placed in sealed Romicron slides and allowed to remain in the refriger
tor several weeks, so that viability could be easily tested by returning the cel
to 26° C.

A serious drawback with the cultures of "normal" kidney, quite contrary
proliferation of tumor acini, is that the origin of the normal series of cells
uncertain. Probably, the luxuriant growths of the normal series are fro
kidney capsule epithelium derived from peritoneum, rather than from th
tubule walls themselves, although this desirable result may have occurre
While it is true that normal cells frequently can be distinguished from mali
nant ones in the tumor cultures, we have not been able to identify *in vitro* an
provable proliferation from a specific normal tubule despite several years
intensive search. It will be recognized that this lack of a true norm is a stan
ard difficulty in all tissue cultures generally not started from a single cell
known origin. It does not affect the validity of our conclusions, but it do
limit them. Until a method is found to induce unaltered kidney tubules
undergo mitosis, a perfect homologue for the malignant cell is lacking. In tw
sets of experiments, an alternative plan was adopted; *i.e.*, macerating the no
mal kidney gently in a hanging drop of culture fluid with microneedles, an
following the changes in torn cylinders of tubules exposed to tumor filtrat
heated tumor filtrate (60° C., 10 minutes), and filtrate from normal kidne
brei.

Feulgen stains of cultures were routinely made after fixation in Kahle's fluid,* with counterstaining with light green. For comparison with both Feulgen-stained cultures and sectioned tissues from the animal, it was found desirable for each series to have at least two additional cultures stained with standard hematoxylin-eosin after fixation in either Bouin's or Tellyesniczky's fluids. It cannot be emphasized too strongly that comparison of the same culture fixed and stained after weeks of observation of it in the living condition is necessary for adequate comprehension of all cytological detail. Data in this category derive from detailed oil-immersion objective studies of about 150 stained preparations, following biweekly observation under phase contrast of approximately 1500 cultures carried on from 4 to 20 weeks but, in one block of experiments, all derived from a single adenocarcinoma of a male frog or one of the three tumors induced by its filtrate.

Results

The data covered in this paper may be grouped and analyzed in the following sequence: (1) production of tumors from injected suspensions of whole cells (tumor brei); (2) the evidence for precancer in histological sections of tumor-filtrate injected kidneys; (3) the relationship of experimentally produced changes to the spreading of spontaneous malignancies through the host kidney; (4) parallel precancerous criteria as observed in tissue culture; and (5) altered nucleolar physiology as the chief presumptive cause for anaplasia and malignancy.

Brei experiments. In a preliminary attempt to provide a laboratory reservoir of cells for tumor experiments derived from a single animal, a female *Rana pipiens* was sacrificed and found to have bilateral tumors, approximately 1 × 2 cm., in each kidney. Half of one adenocarcinoma was removed and ground in sterile Ringer's solution, while the other half was fixed for sectioning. A fragment of the apparently normal kidney area was excised for starting tissue cultures (series F-3) from "infected" cells. Coarsely ground brei of suspended cells was placed in a syringe and 0.1 ml. aliquots were injected into three male and two female frogs. Injections were made bilaterally. Two animals died within two weeks. Two males remained normal until autopsy seven months later, but the remaining female, at the same time, was found to have bilateral tumors, approximately 18 × 19 × 12 mm. in each kidney, with possible metastasis to the lung. A similar portion of this implanted tumor was ground, as before, and injected into the kidneys of two more females. Three and one-half months later, one animal was negative, showing only scars in the kidney, but the other had developed small tumors approximately 2 × 4 × 3 mm. in each kidney. Although it was not possible to continue this strain of tumors, the experiment showed that, while the method was feasible, certain frogs tend to combat injected tumor brei either by lymphocytes, by phagocytosis, or by antibodies, or by combinations of the three.

Tumor filtrates. In the next series, it was decided to carry on the tumor line by filtrates of a tumor containing the presumable virus postulated, but not proved, by Lucké. From TABLE 1 may be seen the over-all results in thi group. Since tumors were obtained from such filtrates as, in turn, were use to produce adenocarcinoma, it was considered that Koch's postulate was fu filled in the sense that the active principle was recovered and used successfull again. As pointed out earlier, approximately 17 per cent of the hosts develope histologically provable adenocarcinoma, nearly 44 per cent did not respon and therefore demonstrate that some frogs have a pronounced immunity t the filterable agent (or presumed virus). Of possibly greater interest are th remaining 39 per cent, which showed pronounced pathological kidney change It was in these latter animals that evidence for precancerous transformatio kidney tubules was found.

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* Kahle's fluid: 95 per cent ethyl alcohol, 16 parts; formalin, 6 parts; glacial acetic acid, 1 part; distilled water, 30 parts.

Brief descriptions of four series will illustrate the types of changes observe

Series 2. Injections were placed in right kidneys only. Six and one-ha weeks later, three animals were autopsied. Frog A, female, showed righ kidney reflected back on itself, with an unidentified lesion. Frog B, femal had developed a 2-mm. tumor near the subcardinal plexus junction in the lef kidney. Histological sections showed definitive adenocarcinoma in this are with numerous adjacent areas of inflammation. In frog C, male, a possib cluster of transformed tubules near end of right kidney was classed as pre cancer.

Series 3. Injections were placed in right kidney of 13 males. One frog die on the sixth day. Survivors were sacrificed at end of two months. Six frog showed definite criteria of precancer in both kidneys, including mitoses an enlargement of tubules, with thickened walls, and increased basophilia. Tw frogs showed heavily involved nucleoli in many tubules, and some section had large inclusion bodies in tubule cells. In those frogs classed as negativ all showed slight basophilia but without other striking changes.

Series 5. This consisted of filtrate injections in left kidneys of 17 male carried 3 months. Four *Triturus pyrrhogaster* were injected in a manner simila

TABLE 1

INCIDENCE OF ADENOCARCINOMA AND PRECANCER CHANGES IN FROGS FOLLOWING INJECTIONS OF TUMOR FILTRATE

Series	Number injected	Months postinjection	Approx. volume of tumor	Positive	Some pre-cancer	Nega-tive	Per cen positive
1	6	7	250–540 mm.^{3}	2	3	1	33
2	3	1.5	5	1	2	0	33
3	12	2	0	0	6	6	0
4	6	2	8	1	3	2	17
5	17	3	9	2	5	10	12
6	8	1.4–3.5	350	3	1	4	38
(7)	—	—	(added to cultures of normal cells)	—	+	—	—
8	2	3	0	0	1	1	0
Totals.	54	—	—	9	21	24	
Per cent.				17	39	44	

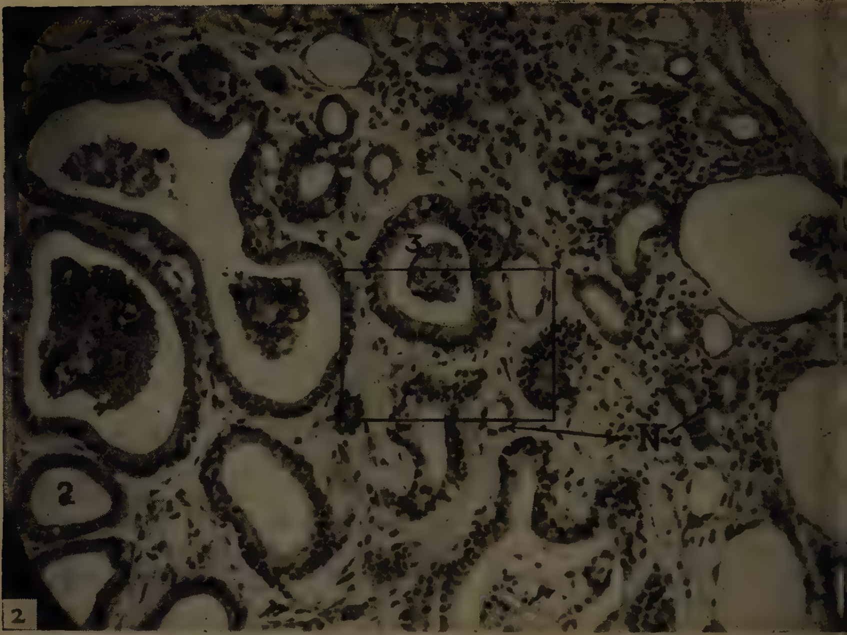
to frogs, but showed no kidney response. In both positive frogs, tumors appeared in the right kidneys, one as a distinct 2-mm. diameter nodule, and the other as two separate nodules about 1.5 and 1.0 mm. respectively. Histological precancer changes in this series were particularly instructive. Of the five classed as precancerous, tubule transformations were beginning in all. Changes included refractive bodies in nucleoli, greatly enlarged nucleoli, mitoses, inclusion bodies in cytoplasm, change in cell shape, and enlarged, infolded tubules. Particular attention is drawn to the fact that, adjacent to the two areas of unequivocal adenocarcinoma, all of these precancerous criteria could be found in abundance.

Series 6. Tumor filtrate injections were placed in the left kidneys of eight male frogs. At seven and one-half weeks, frog A, male, had developed advanced highly malignant adenocarcinoma of both kidneys, which were double normal size. Spread had occurred both by invasion and by infection. A tumor of the left anterior third digit was also noted. Histological examination showed many areas of normal tubules crushed between growing acini. Precancer changes, including nucleolar enlargement and inclusion bodies, were found in these transitional areas. From this tumor, tissue cultures (series FT-23) were carried four months. Frog B yielded a tumor of low malignancy in the posterior half of the right kidney, approximately 350 $mm.^3$, with a double-length left kidney after three and one-half months. From this tumor were made six extended subcultures designated series FT-24-F, from which photographs are shown in FIGURES 6 to 13. Frog C, male, developed a 1.7 gm. low-grade tumor at the anterior end of the right kidney, with a smaller lesion at the mid-line toward the aorta. From the last, a filtrate was prepared that succeeded in inducing advanced precancer grading into adenocarcinoma in a new host. A second host, however, was negative. These latter animals are included in TABLE 1 as series 8.

Spreading Phenomena in the Kidney

Photomicrographs scarcely can do justice to the extent and qualities of renal precancer. Various criteria of precancer changes are only partly evident in FIGURES 2, 3, 4, and 5. FIGURES 2 and 3 are examples of typical transitional areas in a frog with spontaneous adenocarcinoma. To show the close identity of experimentally produced changes by tumor filtrate with the spontaneous transitions, FIGURES 4 and 5 are offered. In all four figures, normal appearing tubules are designated with the letter N, while arbitrary degrees of abnormality are indicated by the numerals 1, 2, and 3 in increasing order of malignancy.

In the low-power view given in FIGURE 2, it is clear that pathogenesis of spontaneous adenocarcinoma involves not only growth by tissue invasion but also by *transformations* of pre-existing structures and cells. The early acini shown here may be reconstructed from the sections. Their interconnections suggest strongly that they are conversions of a proximal tubule leading from a swollen glomerulus, similar to the one shown at the right. Numbers 2 and 3 indicate areas where a second and third degree of transformation have occurred.



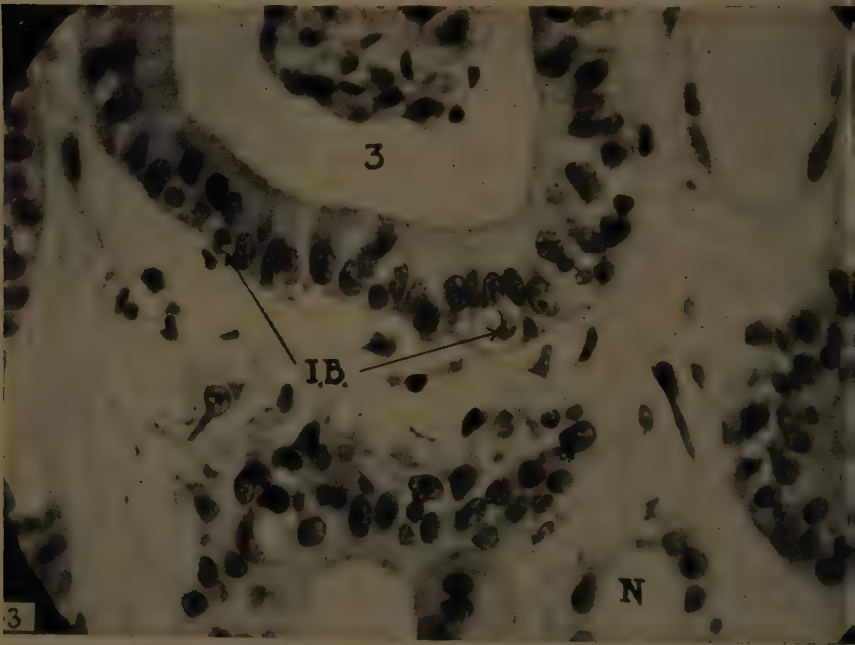


PLATE II

FIGURE 2. Section through transitional area between large spontaneous tumor and remaining functio
frog kidney tubules. Normal tubules (N), stages of precancer indicated by *2* and *3*. Note swollen glomerulus
right, and transforming proximal tubules grading into adenocarcinoma. *Tellyesniczky's, H and E.* Appr
mate magnification: X128.

FIGURE 3. High power view of central area in FIGURE 2. Normal size tubule cross-section (N). Stag
precancer (3) shows inclusion bodies (I. B.), increased basophilia in columnar cells of transforming tubule. S
ernumerary layers of nuclei with enlarged and duplicating nucleoli are evident. Note examples of nuclear sec
tion. ×530.

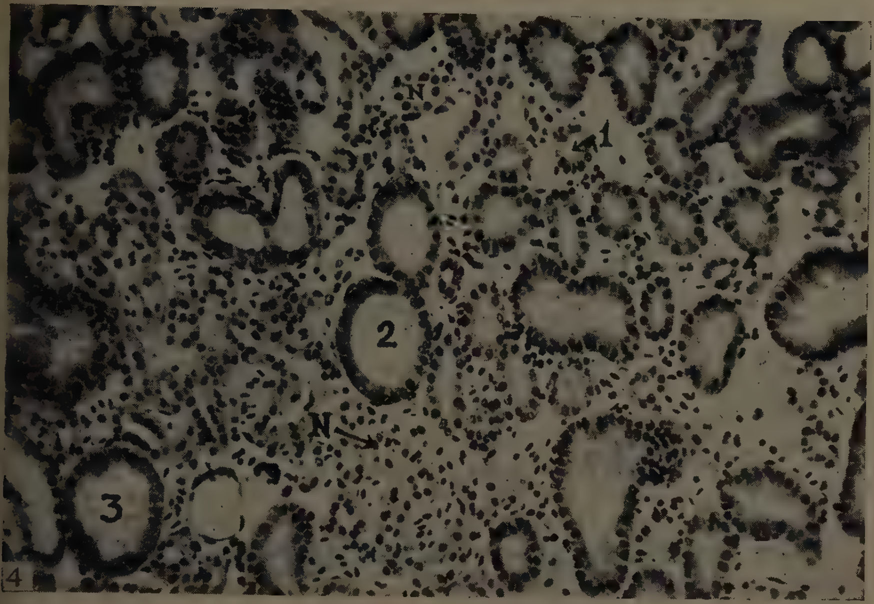


Plate III

Figure 4. Section through frog kidney 88 days postinjection of tumor filtrate. Transitional area approaching induced adenocarcinoma. Three stages of transforming proximal tubules designated as precancerous. Normal tubules (N). Note increased basophilia, altered cell shapes, and approach to definitive carcinoma with columnar secretory epithelium. *Tellyesniczky's, H and E.* Approximate magnification: ×166.

Figure 5. High-power view of section near induced tumor. Normal tubule (N). Two stages of precancer tubule transformations. Note enlarged hyperactive nucleoli, thickened cells, and beginning supernumerary layer, illustrating last stages of normality, 52 days postinjection Approximate magnification: ×700.

In FIGURE 3, the third stage areas is shown under high magnification for com parison with a normal-size tubule cross section (N). This photograph recor change in cell shape, increase in basophilia, development of inclusion bodi (I. B.), and many examples of enlarged, hyperactive nucleoli.

With close reference to FIGURES 2, 3, 4, and 5, especially to the high-pow photomicrographs (FIGURES 3 and 5), a recapitulation of the criteria of pr cancer will be useful. It must be cautioned again that, taken alone, none these criteria is certain but that, when found together, they become mo valid signs of premalignant pathology.

The first or primary criterion of abnormality is nucleolar involvement. Ev dences of hyperactivity are double or triple size, multiple branching, and dro lets (nucleolini). As found earlier, the nucleolar contents are accumulate and enlarged, and then extruded through the nuclear membrane, when r cycling may take place. Consequently, not every nucleolus in a section show the same size (compare FIGURE 8).

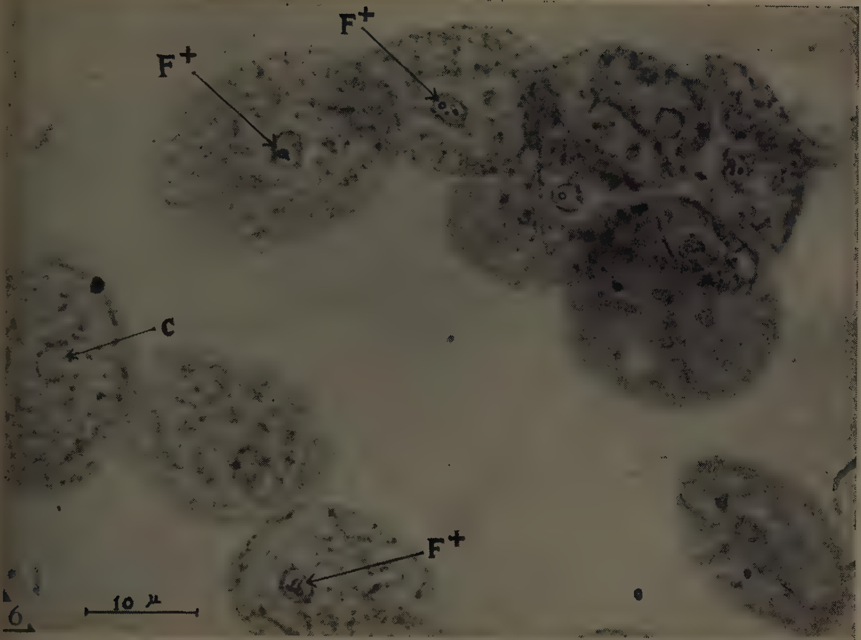
A second criterion is the appearance of oval or branching acidophilic inclu sion bodies in some of the enlarged tubule cells. These irregular bodies, im perfectly described by Lucké, are found in a cytoplasmic vacuole surrounde by a halo. With close examination under oil immersion, an appreciab percentage (from 1 to 10 per cent) usually can be observed in various stages expulsion from the nucleus. In the original photograph for FIGURE 3, ni examples can be found.

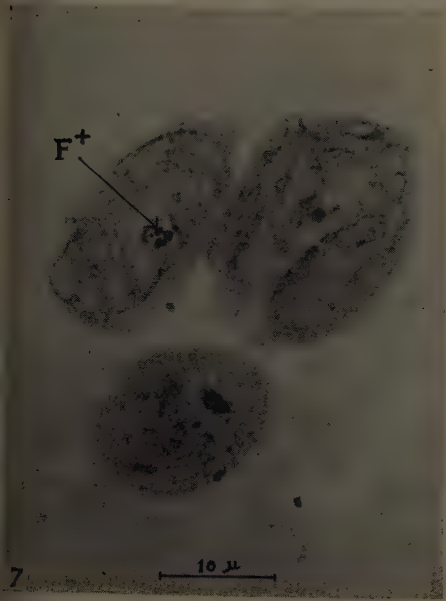
The inclusion bodies characteristic of adenocarcinoma are semisolid gela inous masses. With microneedles, they could be dissected out of both fixe and stained preparations and from living tumor cells. Some inclusion bodie such as the one (I. B.) shown in FIGURES 11, 12, and 13 could be cut apart wit microneedles. Other types were more soluble and were adsorbed into t cytoplasm.

Third, change in cell shape and structure of the tubule wall is both significan and extremely prominent, as FIGURES 2 and 4 show. In FIGURE 4, three stage of transition have been indicated with numbers for comparison with a norma tubule (N). Concurrent increase in basophilia in developing columnar ep thelium is evident. The reader should keep in mind the well-known fact tha in other types of cells, increased cytoplasmic basophilia is associated with hig RNA content.

In fourth place, in physiological sequence, is the appearance of mitosis i the nephron walls. Alone, this is *prima facie* evidence of hyperplasia, sinc normal kidney tubules of adult frogs are uniformly and classically free fro mitosis. High and low mitotic counts were correlated with similar degrees malignancy and metastasis in two instances.

Fifth and last in sequence is infolding of tubule walls resulting from later pressures exerted by addition of new cells. Examples illustrating differen degrees of infolding may be seen in FIGURES 2 and 4. Where other criteria precancer exist, infolding of columnar cell tubule walls excludes completely diagnosis of "normal." It substantiates the impression of unequivocal aden carcinoma to an extent where a panel of pathologists might begin to agre





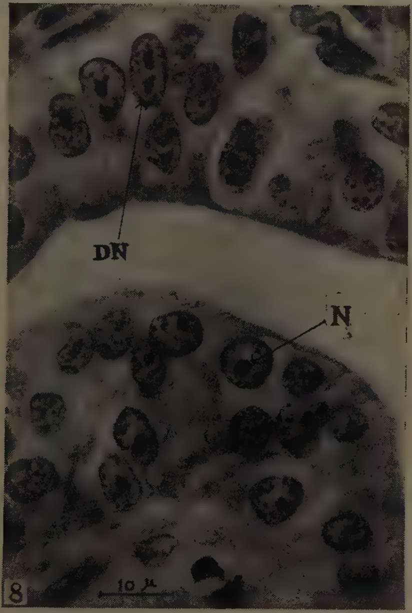


Plate IV

Figure 6. Multinucleate cell from 61-day tissue culture of induced frog renal adenocarcinoma. Of 10 nuclei visible, note that 6 show abnormal DNA in nucleoli. Feulgen-positive granules (F$^+$) in nucleolus centers take forms of droplets, rods, ribbons, chains, *etc.*, typical of coagulated colloids. They are evidence of nucleolar hyperactivity. Nucleolus (C), however, shows typical normal arrangement of DNA capsule around Feulgen-negative center, indicating that not all nuclei of a tumor cell need be virus infected. Compare with figure 9. *Kahle's, Feulgen-light green.* Oil-immersion.

Figure 7. Another trinucleate cell from same culture (FT-24-F-8) as figure 6. Note Feulgen-positive droplets (F$^+$) in nucleolus center. Other nuclei show diffuse type of Feulgen-positive material in nucleoli. Oil-mmersion.

Figure 8. Section through metastasized nodule of frog renal adenocarcinoma in lung. Typical nucleolar nvolvement indicating possible virus activity. Giant nucleolus (N), doubled nucleolus (DN). Many nuclei secreting eosinophilic material into cytoplasm. *Tellyesniczky's, H. and E.*

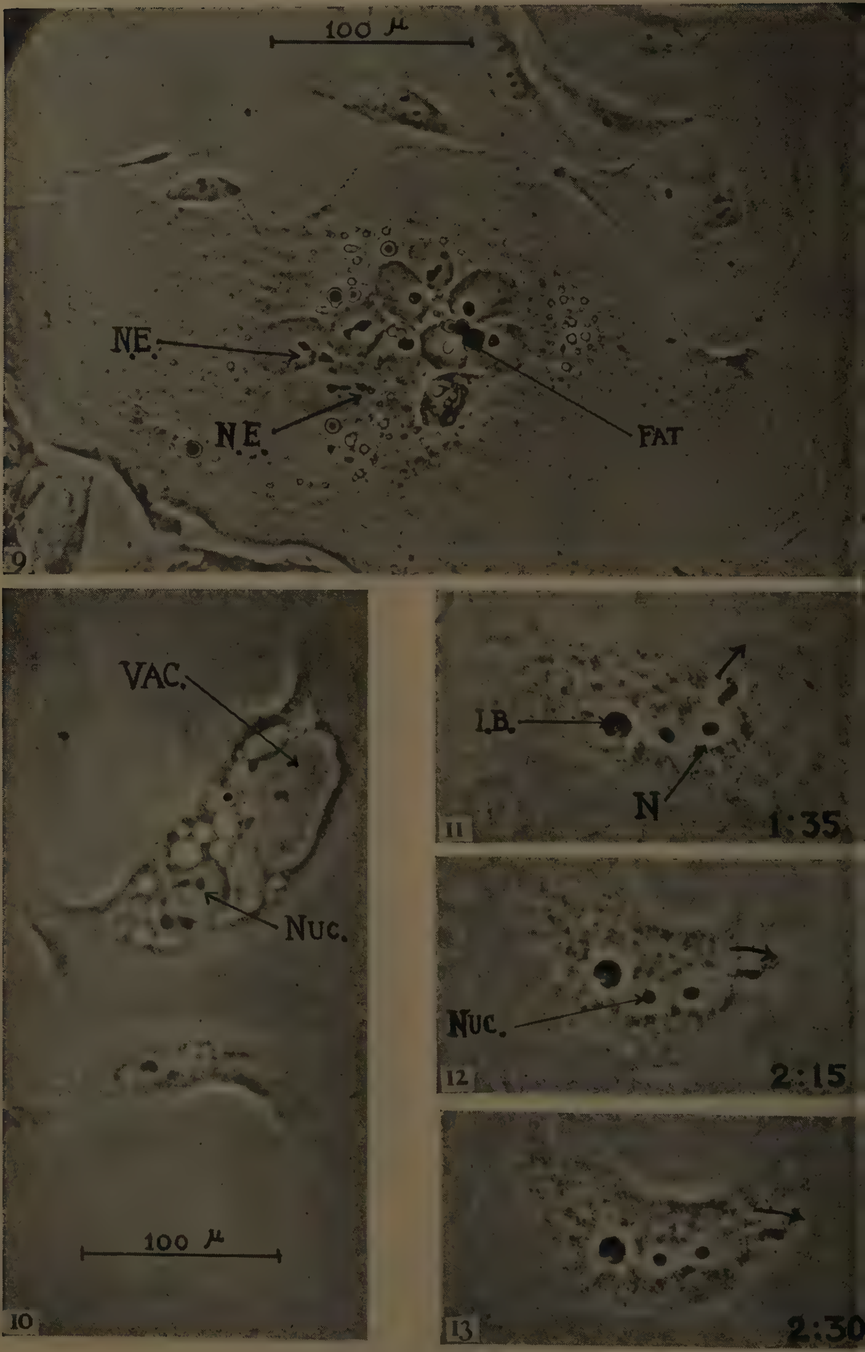


Plate V

To this criterion should be added culture characteristics *in vitro*. At this stage, explanted tissue in question will produce branched cords of acinar cells in liquid medium. Earlier stages have not yet been characterized by the culture method.

As illustrated by comparison of FIGURES 2, 3, 4, and 5, and from a summary of the complete data, the conclusion has been drawn that experimentally induced and spontaneous tumors are so much alike in histological detail that they are microscopically indistinguishable. It is also clear from analysis that experimentally produced tumors spread through host kidneys by invasive growth as well as by infection. Spontaneous tumors give every indication of spreading through the kidney in the same way.

Tissue Culture Experiments

Excised fragments of both tumors and normal kidney, when planted in liquid media under cellophane, reorganized in two to three days by the usual output of vigorous new sheets of cells. To the former were frequently added outgrowths of long solid cords of cells twisting and branching over an area as great as 2 mm.^{2} Lumens frequently appeared in the solid cords reminiscent of the tubule pattern. Wherever the columnar cells of cords adhered sufficiently to the coverslip, squamous sheets of adenocarcinoma spread in monocellular layers, offering exceptional clarity for viewing nuclear and cytoplasmic detail. If sheets were loosened with a microneedle, the columnar acinar cord pattern was immediately resumed. As mentioned earlier, heavy sheets grow out from normal kidney, but these never formed cordlike structures. On the other hand, normal kidney epithelium in about one fifth of the cultures developed active cilia at random places at any time from 5 to 40 days. Very infrequently, cilia differentiated in the tumor cultures, probably from residual fragments of peritoneum. The main advantage of beating cilia is the indication of healthy culture conditions. In order to test precancer responses in normal epithelium exposed to tumor filtrate or tumor brei, it was first necessary to learn the characteristics of each type of cell seen in our cultures and their expected variabilities. In addition to notes and drawings made in the biweekly observations, it was deemed advisable to compile an atlas of about 500 microphotographs in the form of 8- × 10-inch prints, only a few of which can be included here. Most types of cells observed in the sections, except tubule ones, were recognizable in early explant outgrowths; *e.g.*, acinar, epithelial, secretory, adrenal, blood, connective tissue, and giant ones. In the course of weeks, populations tended to become homogeneous due to overgrowth

FIGURE 9. Large multinucleate tumor cell in culture. Four nucleoli in hyperactive state. Nucleolar extrusion (N.E.) through membrane visible at two points. Fat droplets not connected or derived from nuclei (FT-24-F-5B). Compare surrounding average size tumor cells. Fixation: 2 per cent osmic acid, 21× phase-contrast objective (FIGURES 9, 10, 11, 12, and 13 are same magnification).

FIGURE 10. Living adenocarcinoma cells in culture (FT-24-F-B-1). Enlarged cell with vacuole (Vac.) shows giant nucleolus spinning out into ribbon prior to nuclear extrusion. Average tumor cell with small typical inclusion body emerging from nucleus below.

FIGURES 11, 12, and 13. Sequence photographs of nuclear secretion. Typical adenocarcinoma cell in same culture as FIGURE 10. Inclusion body (I.B.) was earlier secreted by left-hand nucleus. On the right, inclusion body may be seen emerging through the nuclear membrane. (N). In FIGURE 12, left-hand nucleolus has distinct satellite, probably marking chromosome attachment point (Nuc.). Arrow shows path of emerging inclusion body through cytoplasm.

FIGURE 13 taken 15 minutes later. Arrow shows further progress of inclusion body away from nucleus. This cell, 12 hours later, showed right-hand inclusion body further out in cytoplasm, while left-hand appeared breaking apart for absorption into cytoplasm; 21× phase-contrast objective. Cells were 71 days in culture, following 55 days refrigeration of excised, filtrate-induced frog tumor.

of the two dominant cell types: adenocarcinoma and the undetermined spec
of normal kidney epithelium.

The following observations on living tumor cells in culture, when weigh
together, conveniently distinguished them from "normal" cells. They we
thought to be only of general use on about four tests out of five, like the earlie
mentioned criteria of precancer that they overlap. It should be kept in mi
that long-continued observation under high-power phase-contrast objectiv
was essential.

(1) Typically irregular size and shape of nuclei are illustrated in FIGUR
9 to 13, since photographic magnification is the same in all cases. Character
tic variations from 15 to 40 μ total diameters can be noted. A typical narrow
variation between nuclei of a single giant cell is clear in FIGURE 9. Norm
cells always showed more uniform nuclei.

(2) Larger (averaging 5 μ), irregular nucleoli, frequently with multiple r
fractive droplets, were fairly diagnostic. Warren Lewis in 1943 noted up
60 per malignant rat nucleolus. The nucleolus often exhibited slow *pulsatio*
of 20 to 30 second durations at 26° C., stretching into flexible bands as illu
trated strikingly in FIGURE 10, or fragmenting and extruding through th
nuclear membranes as seen in FIGURES 9 and 11. Such larger pulsations
cancerous and precancerous nucleoli might be expected as a result of inten
molecular activity that gave rise to local differences in surface tension. Th
are understandable in the light of the writer's demonstration in 1941 that n
cleoli of ovarian eggs are composed of coacervate droplets capable of pha
reversal.

(3) Of great value was the presence of large (10 to 30 μ) cytoplasmic inclusi
bodies first described by the author (1954). These masses, of yet undete
mined character, could be seen to originate inside the nucleus and to be pa
sively extruded through the nuclear membrane. During the extrusion, th
nucleus was apt to *rotate* (*cf.* Pomerat, 1954). Sequence photographs in FI
URES 11, 12, and 13 illustrate this process occurring in about an hour. Sever
hundreds of cells showed the cytoplasmic inclusion bodies originated from
coalescence and expansion of nucleolar droplets. With prolonged reexamin
tion, it was observed that identical processes occurred in living normal cell
but far less frequently and to a much smaller extent. Confirmation of "nuclea
pump" action has been found in many hundreds and in varied types of somat
cells, both of frogs and salamanders. Consequently, this action appears to be
process of major importance in cell physiology. It is best demonstrated wit
15-minute-interval sequence photomicrographs. Inclusion body formatio
should not be confused with basophilic changes or with renal droplet formatio
as seen in sections. For a fuller discussion of this process and of its relation t
similar processes described by other investigators in developing oocytes, re
rence should be made to our earlier paper (Duryee and Doherty, 1954). Fu
ther descriptions are reserved for publication elsewhere.

(4) Another criterion was the appearance of giant cells in a majority of cu
tures that had been carried nine days or more. Sizes of these interesting cel
varied from 200 to 700 μ in diameter. Tumor cells were not classed as "giants
until over 200 μ, since the usual malignant cell was of the order 70 to 100 μ i

the long axis, with frequent examples up to 150 μ. Giant cells were almost always multinucleate, with extremes from 3 to 55 nuclei per cell. Normal giant cells appeared toward the periphery of the cultures, suggesting a preferential response to conditions at the inner edges of the hanging drops covered with perforated cellophane squares. They were convenient for microdissection. At first it was thought that they were completely abnormal, but comparable smaller examples have occasionally been found in sections of spontaneous tumors in the frog.

Turning next to fixed and stained tissue cultures, we may add to the list two other possible criteria for tumorous and precancer changes.

These are (FIGURE 7) a *diffuse* Feulgen-positive coloration of the nucleolus center and (FIGURE 6) a sharply distinct complex of Feulgen-positive droplets or granules within the nucleolus itself. The former, a diffuse coloration, may be detected by critical oil-immersion microscopy in practically all nucleoli of malignant origin, and in some nucleoli of normal cells exposed to tumor filtrates. In contrast, the nucleolus center of normal cells is usually Feulgen-negative and takes a clear green coloration after counterstaining with light green. These conditions, being diagnostic criteria, of course are usually observed separately in different cells. They very rarely occur together, however, in the same multinucleate malignant cell as illustrated in FIGURE 6, and the fact that they may occur together in adjacent or in the same cells is extremely instructive.

Nuclear Physiology

Since diffuse Feulgen-positive coloration of nucleolar centers is likewise characteristic of spontaneous adenocarcinoma in histological sections, the conclusion is warranted that, so far, it is the best and most fundamental indication of the cancerous condition. Concerning the more striking and more easily observed pleoform Feulgen-positive granules, there is still some doubt. Because of their relatively large measurable size (0.5 to 1.0 μ), it is unlikely that they directly represent the tumor virus. Also, in 7 out of 20 normal cultures stained with Feulgen reagent, there were positive granules, but of smaller and different character. Excellent examples are shown in FIGURES 6 and 7. It will be noted that they may take the form of rings or clumped chains. The fixed, stained, and hence coagulated examples from adenocarcinoma cultures had such variable patterns ranging from droplets within droplets to chains, ribbons, clumps, and fragments, that one might well conclude that they represent products of extremely active sites of DNA metabolism. In the few instances where refractile or DNA-positive granules were observed in cultures of "normal" epithelium, they were more uniform and associated with the nucleolar shell or capsule. An example of this more normal type of capsule characteristic for normal epithelium, although here in a malignant cell, is indicated by the arrow *C* at the left in FIGURE 6. The conclusion is quite clear that, even in tumor cells, not all nucleolar DNA is abnormal. If one accepts the virus hypothesis, it can be postulated that tumor virus particles do not necessarily infect all the nuclei even of one cell and, by the same token, they do not infect all the nuclei of adjacent cells in one tubule or of one kidney.

Comparisons were made between cultures from a highly malignant tumo (*e.g.* series FT-19, where the parent tumor had heavily metastasized) and thos from a "low-grade" tumor that had been hampered further by keeping ther for 38 days at 6° C. In the former group, Feulgen-positive granules increase to a maximum in more than half of the cells between the 16th and 48th day of culture, after which they slowly diminished in numbers. In contrast, n culture in the latter series (FT-24-F) had more than 30 per cent of the nucl infected. One particular giant cell with 27 nuclei exhibited only one nucleolu infected with only a single Feulgen-positive particle. This is comparable t the aberrant normals, where infected nucleoli were always below 10 per cent.

Of special interest to report here were the findings in culture series F- taken from a "normal" kidney residual tab adherent to a large tumor. Sections showed this area had acquired some precancerous changes in the stil functioning tubules. Cultures of tissue from this area exhibited an abnormall large percentage of nucleoli with Feulgen-positive centers. Giant cells appeared by the 30th day, and large inclusion bodies were observed in some cell These results in cultures are consistent with the hypothesis that a carcinogeni infection had taken place in residual kidney epithelium while a large tumor wa developing nearby.

Preliminary experiments to test response of macerated tubule segments t tumor filtrate were set up by diluting standard medium with filtrate in th proportion of 10:1. In the F-8 series, at the end of 24 hours, some cylinder showed evidence of cell clusters on the surface and some nucleolar change Observations in segments were difficult and changes less clear than in monocellular sheets, so this series was discontinued. Two other culture series wer established by first growing large sheets from normal kidneys and then separating cultures into four groups: (1) untreated; (2) received dilute tumor filtrate (3) received heated tumor filtrate (60° C., 10 minutes); (4) received dilut filtrate of normal kidney. In one set, following a single exposure to filtrate various degrees of response were noted by the 11th day in group B, but not i the other groups. These were: (a) enlarged, irregular nucleoli, with frequen internal reduplication; (b) increase in numbers of refractive granules per nucleolus; (c) appearance of actively ameboid giant cells; (d) massive inclusio bodies (20 μ) in cytoplasm of some cells; (e) greater spacing between cell with less tendency to form continuous sheets. In another series, no result were noted by the 12th day, so a second drop of stored filtrate was added t group B cultures. By the 14th day, some of the above changes were definite but the results were inconclusive. Their pathological significance could no be determined readily, so further experiments were postponed until more frequent exposures to freshly prepared tumor filtrate could be made. It wa concluded, however, that the filterable agent could elicit nucleolar changes although the significance was still uncertain.

Discussion

The establishment of a filterable agent as the inducer of renal adenocarcinom lends strong support to the virus theory of Lucké. But an even greater valu of the experiments lies in their clear indication of pathogenesis by infectio nd transformation of proximal kidney tubules. The evidence is against an ll-or-none phenomenon. It favors an exogenous catalyst concept.

It is beyond the scope of this paper to evaluate the biochemical composition f the presumed virus or the DNA or RNA content of the filtrate. Some other raction of the filtrate could be the actual agent. Nevertheless, there is a arge and growing literature on the chemistry of other animal viruses that are ccepted as the nucleic acid component of nucleoprotein and, in some instances, s DNA itself. The role of RNA in many plant or animal viruses is far from eing settled. Further support for the exogenous catalyst concept comes from he parallel findings of Cohen, Levi-Montalcini, and Hamburger (1954). These nvestigators reported that extracts of mouse sarcoma 37 and 180 contain a owerful growth-stimulating factor in the nucleic acid fraction of the microomes. Their partly purified residue contained 26 per cent RNA and 0.3 per ent DNA, but was not found in the nuclear fraction. Attention is likewise irected to the work of Stewart (1955), who found that extracts and filtrates f mouse leukemic tissues induced parotid and adrenal gland tumors. It is mazing that in a recent review of interaction of virus and animal cells (Dulecco, 1955) almost no mention is made of amphibian adenocarcinoma. That uthor agrees with Lucké that there may be some "specific interaction between he virus and some cell constituents," but disagrees with Rose's theory (1952) of irus transformation through host passage. Our observation that a small perentage of spontaneous renal tumor frogs also carry sarcomalike tumors of the igits and fat bodies suggests passenger virus transmissions to explain Rose's esults, rather than transformations.

With full warning that the following discussion of mechanisms is largely peculative, it is hoped that the points it raises may lead to decisive experiients. From the time of Boveri, we have known that malignancy was inariably associated with some form of nuclear abnormality. Numerous inestigators, including Warren Lewis (1943), have called attention to nucleolar nlargements involved in some unspecified manner with certain types of ialignancies. In the third paper of this volume, Cowdry (1956) listed about 1 properties of cancer cells, including "active protein synthesis, indicated by ntense ribonuclease activity and large nucleoli, monopolizing nutritive marials needed by all other cells." Could Cowdry's list be too long? If the irus theory is reduced to organ specific terms and granted endogenous properes also comparable in some regards to *Escherichia coli* phages under special onditions such as irradiation or chemical shock, do we need to look further? *he conclusion now seems warranted that, until some more promising theory is lvanced, frog renal adenocarcinoma, in its pathogenesis, is a virus disease of the ll nucleolus.*

In the experiments reported here, abnormal DNA (including Feulgen-posive bodies) has been shown to invade the nucleolar cores and to influence the ajor RNA producing organoid of the cell. Hyperactivity of the nucleolus dicated by enlargement, multiplication, and more rapid pulsation has resulted hypersecretion by the nucleus exposed to tumor filtrate. Hypersecretion is ident in sections of kidneys of injected frogs that have developed adenorcinoma and in many cases of those sacrificed early, which showed inter-

mediate conditions designated as precancer. The resultant growth and mit
clearly depends on active protein synthesis, agreeing with Cowdry's ma
point above. The reader's attention is called to the striking parallelism
nucleolar changes in human neoplastic cells reported elsewhere in this mo
graph by Margaret E. Long (Long and Taylor, 1956). From an entir
different quarter comes support for the view that the nucleolus is essent
for protein synthesis. Lindsay and Barr (1955) have demonstrated that,
cat nerve cells depleted of Nissl material by prolonged stimulation, the nucleo
swells and vacuolates during restoration by synthesis of ribose-nucleoprotei
In the sections of frog kidneys showing precancer tubule transformatio
basophilia appears early and increases with degrees of change to malignan
This correlates with the concept that *new protein synthesis* is the key chemi
process involved. The importance of nuclear DNA in protein synthesis
thymus cells is now partly understood (Allfrey, 1954), and new informati
on RNA in relation to cytoplasmic protein is forthcoming. What is not kno
is whether the new protein is a normal one for the cell, produced to an abnorm
degree, or whether the new protein is of some specific pathological type, th
begging the question. At this time, however, the experiments reported he
fit better with the former alternative.

Transformed tubule cells behaved as though "goaded" by a stimulant
secrete and divide in their determined fashions, but to an *abnormal* degr
The extended period of time involved suggests that host cells offer resista
to the presumed virus. In terms of the theory, it could be said that nucleol
infection and derangement in 44 per cent of injected animals was not achieve
Reference might again be made to FIGURE 6, where not all nuclei in one c
have been infected, and to the cultures of low-grade malignancy (series F
24-F), where less than 30 per cent of the cells showed Feulgen-positive bodi
It appears that continued presence of the exogenous catalyst is necessa
Most of the changes in tumor acinar growth can be accounted for as extre
and abnormal degrees of normal processes; *i.e.*, in the case of kidney tubul
the infolding phenomena being due to increased cell numbers and the typic
swelling or ballooning of the acini being due to abnormal rates of secreti
Interesting questions regarding the mechanisms of host resistance may
raised, especially because, in this area of natural immunity, unexplored mea
for cancer prevention and possible therapy exist. It would be particular
desirable to know if and how regression occurs. Thus far, it has not be
observed. For definitive answers to many of these problems, new micro-to
and single-cell transplantations of nucleoli and inclusion bodies will be nece
sary, such as those envisioned by Kopac (1956).

Concerning the avenues of infection and spread, there is but slight evidenc
The frog has functional nephrostomes that lead from the coelom to the proxim
tubules. Penetration into the cells should be expected, as there is recent ev
dence for proximal tubules having the ability to absorb proteins as large
hemoglobin (Oliver, MacDowell, and Lee, 1954). Absorption "droplets" se
in their massive dose experiments should not be confused with inclusion bodie
Penetration into the nucleus might be thought a difficulty but, in our cases
cultured kidney epithelium exposed to filtrate, hyperactive nucleoli we

observed by the 11th day, with possible reduplication of diffuse DNA and increase in nuclear-secreted inclusion bodies. There were many examples of Feulgen-positive granules escaping from the nucleus with the inclusion bodies, and a few examples of escape from the cells, but there is still no proof that occasional Feulgen-positive material found between the cells represented virus aggregates. The extreme variety and numbers of these bodies seen in the nucleoli can be interpreted only as fragmentation phenomena, with the smallest visible granule adjacent to a larger droplet being the most common. Our finding that transmission depended on direct injections of filtrate into the kidney, together with Lucké's observations on the organ specificity of the agent, lead to a clear understanding of why cancer does not ordinarily spread from animal to animal.

Recently a most useful study of this tumor has been undertaken by Fawcett (1955), who reported intranuclear inclusion bodies arranged in rows of hollow spheres, thus confirming our earlier light-microscope observations on the presence of intranuclear forms of the virus. In addition, Fawcett's electron micrographs have shown "sheaves of dense filaments" and aggregations of vacuoles, both of unknown origin in the cytoplasm.

For the amphibian kidney, various stages of precancer are now clearly recognizable. A filterable etiologic agent, partly susceptible to heat inactivation, can be recovered from spontaneous tumors. Pathogenesis is gradual, with many cells of a tubule being affected concurrently. There is circumstantial evidence that the changes are progressive and not of the all-or-none variety. Host susceptibility proved to be more important for carcinogenesis than did virulence of the agent. There was no evidence of an "irreversible point" in the process. Indeed, precancer and definitive adenocarcinoma, of both low and high malignancy, arose from the same batch of filtrate. It is most likely, therefore, that the cytological differences between benign and malignant tumors are due to nucleolar changes not of kind, but of degree.

Summary

(1) The etiologic agent for frog renal adenocarcinomas will pass through pores of a filter having a maximum size of 0.85 μ, thus giving strong support to the virus theory of Lucké.

(2) Definable stages of precancer are recognized by (a) hyperactivity of the nucleolar colloid; (b) hypersecretion of nucleolar organized material through the nuclear membrane into the cytoplasm to form inclusion bodies; (c) change in the shape of proximal tubule cells from cuboidal to columnar form accompanied by increased cytoplasmic basophilia; (d) mitosis in the tubule wall and appearance of supernumerary layers; and (e) new growth characteristics in the animal and in tissue culture. None of these criteria is sufficient in itself, but must be considered in relation to the others.

(3) Aqueous tumor filtrates, when injected directly into the kidney of normal frogs (*Rana pipiens*), induced adenocarcinomas in 17 per cent, various degrees of precancer in 39 per cent, and no recognizable changes in 44 per cent.

(4) Pathogenesis was gradual (7 to 10 weeks minimum) with many cells affected concurrently. Progressive transformation of renal tubules into tumor acini could be demonstrated.

(5) Differential host susceptibility was shown more important than deg of virulence in the etiologic agent.

(6) Cytological changes both in tissue sections and tissue cultures from same animals agreed in showing new DNA involvements of the nucleoli, lowing exposure to tumor filtrate. Increased nucleolar size, vacuolizati and activity led to heavy secretion of semisolid inclusion bodies (action c nuclear "pump"). Evidence for a similar but inconspicuous pump action found in normal cells.

(7) A hypothesis for precancer in the frog kidney is offered, based on in tion by an exogenous virus in the nucleoli of proximal tubule cells.

Acknowledgments

For technical assistance in the preparation of tissue cultures and for hel photomicrography, the author wishes to thank Mrs. Josephine K. Dohe To Dr. Howard B. Andervont thanks are due for administrative help and stimulating discussion of the theoretical aspects of carcinogenesis.

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SOME EARLY EFFECTS OF CARCINOGENIC HYDROCARBONS ON MOUSE SKIN*

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In the experimental carcinogenesis of mouse skin, a number of early changes have been described. These have been summarized recently by Cowdry (1953), who himself inspired many detailed investigations in this field. In his review, Cowdry (1953) has stressed the fact that all epidermis exposed to carcinogen is not precancerous, because cancer ultimately develops only in small and restricted areas of the treated skin. Hence, there may be a degree of uncertainty about the necessary involvement of any observed early wide-spread change in the development of neoplasia itself. Nevertheless, some of the changes in mouse epidermis made hyperplastic by the application of carcinogenic hydrocarbon appear to be precursors of even greater changes in the final attainment of the neoplastic state.

Besides the better-known morphological differences between normal epidermis and precancerous hyperplastic epidermis, such as increase in number of cell layers and enlargement of nuclei and nucleoli, some differences in chemical composition have been recorded.

Perhaps the earliest change observed has been the formation of fluorescent derivatives of carcinogenic hydrocarbons, combined chemically with proteins of the epidermis. They are detectable within three hours of painting with 3,4-benzpyrene (Miller, 1951). There is a correlation between carcinogenicity of hydrocarbons and the extent of their binding by mouse skin. Methylcholanthrene, for example, is strongly bound (Heidelberger and Moldenhauer, 1955).

The hydrocarbons are bound to protein, not to nucleic acid (Miller and Miller, 1952; Wiest and Heidelberger, 1952), although conceivably they could be bound to the protein moiety of nucleoproteins. By cell fractionation studies, for instance, dibenzanthracene is found attached to nucleoprotein, particulates, and soluble protein, but not to nucleic acid (Wiest and Heidelberger, 1953), and benzpyrene is found to be bound chiefly to the chromidia (basophilic material) and mitochondria of the epidermis of day-old mice (Fiala, Sproul, and Fiala, 1955). Benzpyrene is not found in the nuclear fraction, which confirms the observation by Simpson and Cramer (1945) that the blue-fluorescent derivative of methylcholanthrene in painted mouse epidermis is not detectable in the nuclei. Supporting evidence on this point is the finding by Zamenhof, Leidy, and Reiner (1954) that methylcholanthrene does not influence the transforming activity of *Hemophilus influenzae* deoxyribonucleic acid, although ultraviolet light and nitrogen mustard readily inactivate it.

Maximum binding of 1,2,5,6-dibenzanthracene-9,10-C^{14} occurs at two

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* The investigation described in this paper was supported in part by a grant from the American Cancer Society, New York, N. Y., and in part by Research Grant C-678(C7) from the National Cancer Institute of the National Institutes of Health, United States Public Health Service, Bethesda, Md.

days (Wiest and Heidelberger, 1953). By this same time after application
the carcinogen, another early and perhaps related change is evident. T
"fast-acting" sterol, Δ^7-cholester-3β-ol, is brought to a minimum in concent
tion by five days after a single painting with methylcholanthrene (Kandut
and Baumann, 1954). Again, the extent of this diminution in Δ^7-cholestero
related to the carcinogenicity of the hydrocarbon that induces it (Kandut
and Baumann, 1955). The drop in Δ^7-cholesterol is most apparent in the e
dermis, but it also occurs in the dermis. Cholesterol is not affected to t
extent, although earlier Wicks and Suntzeff (1945) reported a drop of abo
one half in ratio of cholesterol to protein nitrogen by five days after one pai
ing with methylcholanthrene.

The reduction in the ratio of total lipid to protein nitrogen to 50 per ce
of control values, by the fifth day, earlier reported by Wicks and Suntz
(1942), may be related to the destruction of the sebaceous glands. Simi
changes in the proportions of various cell types, as the mouse epidermis
comes hyperplastic, may account for some of the quantitative changes
chemical constituents of the epidermis (Banyen, 1952). It has been suggest
that this, in part, is the cause of the changes in amount and distribution
free amino acids in hyperplastic epidermis, which comes to resemble the e
dermis of newborn mice more closely in this respect than the normal epider
of adult mice (Roberts, Caldwell, Clowes, Suntzeff, Carruthers, and Cowd
1949).

A change in cell population may account in part for the drop in calcium co
tent of the epidermis observed by Carruthers and Suntzeff (1943). Hyp
plasia of mouse skin resulting from application of methylcholanthrene, scar
red, or croton oil brings the formerly thin epidermis to resemble human e
dermis, for example, with a better-developed spinous layer, and the spino
cells are not so rich in calcium as are the basal cells (Carruthers, 1950). I
letta, Cowdry, and Lischer (1941), however, did observe with microincinerati
a demineralization of the distal portion of the spinous layer in methylch
lanthrene hyperplasia. Epidermis made hyperplastic with methylcholanthre
loses its ability to exchange Ca^{45} actively, probably because of some change
the calcium-binding complex at the cell surface (Lansing, Rosenthal, and K
men, 1948). The decrease in calcium is accompanied by decreased mut
adhesiveness of the cells (Coman, 1947), a property that also distinguish
cells of malignant neoplasms (Coman, 1944).

Together with the drop in calcium, there are decreases in epidermal ir
(Carruthers and Suntzeff, 1942), copper, and zinc (Carruthers and Suntze
1946), and in citric acid (Miller and Carruthers, 1950). Sodium, potassiu
and ascorbic acid show no changes in content under treatment, but magnesiu
is increased (Carruthers and Suntzeff, 1943).

As for enzymes, succinic dehydrogenase activity changes little in meth
cholanthrene-hyperplastic epidermis, and cytochrome oxidase increases sligh
(Carruthers and Suntzeff, 1947). Lipase activity decreases (Kung, 1949) b
cytochrome C shows no change (Carruthers, 1950). Not until carcinoma
velops are there striking increases in activity levels of arginase (Roberts a

rankel, 1949), adenylpyrophosphatase (Roberts and Carruthers, 1948), and uccinic dehydrogenase (Carruthers and Suntzeff, 1947).

There is an increased mitotic activity in the treated epidermis (Cooper and Reller, 1942), evident before 24 hours after application of the carcinogen (Reller and Cooper, 1944; Glucksmann, 1945). In skin regenerating after being wounded, mitotic activity in the epidermis begins when ribonucleic acid increases in the cytoplasm (Firket, 1951). Similarly, in mouse epidermis painted with methylcholanthrene, an increase in cytoplasmic ribonucleic acid may be shown within the first day after painting (Biesele, 1944), by the pyronin-ribonuclease technique of Brachet (1940).

Let us recall the general association of increased cytoplasmic basophilia with ncreased cellular proliferation and, in particular, the observation by Firket (1951), that healing wounds show higher epidermal mitotic rates when moistened with a solution of ribonucleic acid.

Moberger (1954) has applied X-ray absorption and ultraviolet spectrophotometric techniques to frozen-dried mouse epidermis and vaginal epithelium n early stages of carcinogenesis induced by methylcholanthrene, as well as to clinical specimens. His observations reveal a decreased concentration of cytoplasmic proteins, especially in the swelling spinous cells. It should be noted that the water content of epidermis increases under methylcholanthrene reatment (Suntzeff and Carruthers, 1946). Moberger found that, despite the decreased concentration of protein per unit volume, the amount of protein per ell in the spinous layer actually increased, and the cytoplasmic nucleic acid kept pace with it, there being a constant amount of nucleic acid per unit of weight. In carcinomas, the peripheral cell layers of infiltrating cancer cords ontained significantly higher-than-normal concentrations of cytoplasmic nucleic acid. In this respect, Moberger essentially confirmed the findings of Caspersson and Santesson (1942).

All of the foregoing constitutes the basis for a considerable challenge to an lectron-microscopic study of early changes in epidermal carcinogenesis. Perhaps the most promising subject for investigation would be the cytoplasmic particulates, for the mass of evidence above suggests that lipid-ribonucleoprotein complexes, of the sort envisioned by Davidson and Waymouth (1944) re disaggregated under the influence of carcinogenic agents. This disaggregation might be accomplished by the binding of the carcinogenic hydrocarbon to he protein moiety of the complex and its consequent denaturation. This would release the nucleic acid (Kopac, 1947) and, perhaps, the lipide as well. A similar displacement may be involved in the loss of calcium-binding activity t the cell surface. Dissociation of nucleic acid and protein in the cytoplasm might account for greater pyroninophilia, by making the nucleic acid more vailable for dye-binding.

It is of interest, in this connection, that polar-nonpolar compounds such s detergents may be active cocarcinogenic agents (Setälä *et al.*, 1954), and hat Carruthers *et al.* (1955) find the initial binding of a detergent to extracted pidermal protein to alter the molecular structure of the latter, as indicated by change in electrophoretic mobility.

On the other hand, removal of a sterol component from a cytoplasmic lip ribonucleoprotein complex might provide a condition necessary for uncontroll growth envisioned in the theory of carcinogenesis mentioned by Osgood (1955 if the sterol were an inhibitor of synthetic activity in the nucleoprotein, a if the function of the nucleoprotein were autosynthesis or the synthesis of n protoplasmic material necessary for reduplication of the cell. Electron micr scopic examination might reveal an increase in number of small cytoplasm particulates.

In the theory of carcinogenesis of Miller and Miller (1952), the change loss of certain growth-limiting enzymes from cells that keep their proliferati systems intact might result in neoplasia. Essentially, this theory has be presented in expanded form by Rusch (1954). Loss of a cellular mechanis for special differentiated function might permit the mechanism for cellul reduplication to come into action relatively unhampered by competition f available substrates. Electron microscopy could reflect this sequence of even in disintegration of certain cytoplasmic particulates, followed by increase others.

Notable contributions to our understanding of the macromolecular nucle proteins of cytoplasm have been made by Petermann and her collaborators ultracentrifugal and electrophoretic studies. In various types of tissue, the particles appear to be qualitatively similar but to be present in different pr portions and amounts (Petermann *et al.*, 1954). In regenerating liver, the is an increase in the more slowly sedimenting, smaller particles richer in pento nucleic acid, the ratio of C to B particles changing in agreement with the mitot incidence (Petermann *et al.*, 1953). It might be expected, therefore, th electron microscopy of sufficiently high resolution would reveal increases smaller cytoplasmic particles in hyperplastic epidermis.

Cytoplasmic basophilia is currently held to be an expression of the sma submicroscopic particulates of the cytoplasm (Porter, 1954), first described thin sections by Palade (1953). These particles, with diameters of the ord of 80 to 300A, appear to correspond to Petermann's macromolecules. T small particles are often found associated with the endoplasmic reticulum, a rayed on the outer surface of the vesicles, as may be seen in thin sections, or Slautterback (1953) found in liver homogenates. This disposition of the pa ticles is especially common in differentiated cells with secretory function The particles may also be found diffusely scattered in the cytoplasm, with evident association with any other component. The latter disposition especially common in proliferating cells, such as those in embryonic tissue or the basal layer of the epidermis, in which the endoplasmic reticulum is n prominent (Porter, 1954).

This disposition of the small granular component of cytoplasm has also bee noted in neoplastic cells. Thus Porter (1954) described occurrence of the pa ticles in free clusters in the cytoplasm as well as on the surface of endoplasm vesicles in cells of a rat sarcoma. Howatson and Ham (1955a, 1955b), from study of rat liver tumors and adult, regenerating, and embryonic rat liver concluded that the presence of large numbers of the small particles (ergast plasmic granules) accompanies rapid growth of both normal and neoplast cells, while an abundance of organized ergastoplasmic structures (endoplasmic reticulum) characterizes actively functioning differentiated cells.

Just what changes in disposition, abundance, and size of these small particulates could be found by electron microscopy in early stages of epidermal carcinogenesis would be fascinating to determine. Regrettably, a prolonged illness prevented us from carrying out the electron microscopy we had hoped to include in this paper. According to work by Selby (1955), the basal cells of mammalian epidermis contain no endoplasmic reticulum, and the submicroscopic particulates have diameters of 80 to 150A and are associated with no membrane or vesicle in their random placement throughout the cytoplasm.

In the electron microscopic study of experimental epidermal carcinogenesis carried out by von Albertini (1953), no attention was paid to the then-still-undescribed small cytoplasmic particulates or to the fine structure of the cytoplasm beyond the tonofibrils, which became more distinct in early hyperplasia but were absent from the malignant carcinomas.

There have been several oblique suggestions that the small ergastoplasmic granules of the cytoplasm may be derived from somewhat similar granules observed in the nucleolus by electron microscopy (Porter, 1954; Bernhard, Gautier, and Rouiller, 1954). The fullest development of this thesis is that by DeRobertis (1954).

If there is an increase in the small ribonucleoprotein particles in the cytoplasm early in epidermal carcinogenesis, as appears likely from the foregoing, and if these particles are derived, at least in part, from the nucleolus, an explanation is of course at hand for the enlargement of nucleoli seen by the second day after application of carcinogenic hydrocarbons (Page, 1938).

Along with nucleolar enlargement, there is an enlargement of the nuclei (Page, 1938; Pullinger, 1940; Cowdry and Paletta, 1941a; Kraemer, 1946) accompanied by a decrease in intranuclear viscosity (Cowdry and Paletta, 1941b).

By the second day after methylcholanthrene painting, the first instances of enlarged chromosomes were noted in 2 metaphase mitotic figures among 100 examined (Biesele and Cowdry, 1944). They became somewhat more frequent after that, showing up, on an average, in about 10 per cent of the metaphases examined in epidermis treated for 3 to 109 days with as many as 36 paintings of methylcholanthrene in benzene. In epidermal carcinomas of the mouse, the majority of the mitoses prepared for examination by the acetocarmine-squash technique appeared to have chromosomes enlarged beyond those in normal or benzene-treated epidermis.

Because metaphase figures with enlarged chromosomes tended to be paralleled at about twice their frequency by resting nuclei with more than the four nucleoli to be expected from the diploid set of mouse chromosomes, or with more han the number of heterochromatic segments present in diploid resting nuclei of control epidermises, it was concluded that the enlarged chromosomes might be abnormally multiple-stranded (Biesele, 1944). Of course, it is also possible hat the variability in the resting nuclei was partly or wholly occasioned by ncrease in chromosome number.

We have sought clarification of the chromosomal enlargement in early stages

of epidermal carcinogenesis by making use of the improvement in cytologic
technique introduced by Hughes (1952) and by Hsu and Pomerat (1953
namely, the spreading of chromosomes in mitotic figures by hypotonic trea
ment of our tissue cultures.

It was thought better to make explants of skin that had been treated o
the living mouse, rather than to apply carcinogen to tissue cultures. Mill
(1951) did not find the protein-bound fluorescent derivatives of 3,4-benzpyren
after one or two applications of the carcinogen to the skin of freshly killed mic

Experimental Procedure

The animals used were Rockland Farms albino Swiss female mice about
months old, of mean weight 36 grams. The hair was clipped from the midd
back of all mice. The mice of one group were then painted with one brus
stroke of 0.3 per cent methylcholanthrene in benzene, another group wa
painted similarly with benzene alone, and a third group was not painte
Paintings were carried out on Monday, Wednesday, and Friday of each wee

Portions of skin were explanted from 2 methylcholanthrene-painted,
benzene-painted, and 2 unpainted mice at each of the following times: 1 da
after a single painting, 4 days after a single painting, 1 day after 4 painting
over 9 days, 1 day after 5 paintings over 11 days, 1 day after 9 paintings ov
21 days, and 1 day after 15 paintings over 36 days. The animals were paint
at 11 A.M., and they were also sacrificed for tissue culture at 11 A.M. I
addition, two experiments were carried out with newborn Swiss mice. In o
experiment, litter-mates 28 to 30 mm. long were used. In the other, slightl
older litter-mates 32 to 34 mm. long were used. These were given one pain
ing, the animals were placed in separate boxes and, 24 hours later, pieces
skin from the back were explanted.

Plantings were made in plasma clots on coverslip inserts in roller tubes.
fluid medium of Gey's balanced salt solution, chick embryo extract, hors
serum, and human placental serum, standard for this laboratory (Biesele, 195
was used. After two to six days, representative cultures were photographe
under oil immersion with phase contrast, and others were fixed and stained b
various procedures.

For chromosome study, chief use was made of the Hsu-Pomerat (195
hypotonic treatment. The cultures were exposed for half an hour at 37° (
to a mixture of 20 per cent Gey's complete salt solution plus 80 per cent Gey
solution minus sodium chloride. They were then fixed for one hour in alcoho
acetic acid 3:1 and stained with the Feulgen nucleal reaction and light gree
for permanent mounts in diaphane.

Observations

The explants had poor epithelial outgrowth. Most of the outgrowth a
peared to be of mesenchymal, fibroblastlike cells. The newborn skin explan
produced a better outgrowth of epithelium than did the adult skin explan
yet, even with them, it is probable that most or all of the mitoses studied we
in the mesenchymal cells. Hsu (1952) has likewise indicated difficulty in t
culture of adult epidermal cells, albeit from the human subject.

TABLE 1

FREQUENCIES OF CHROMOSOME NUMBERS COUNTED IN TISSUE CULTURES OF ADULT AND NEWBORN MOUSE SKINS EXPOSED TO HYPOTONIC MEDIA.

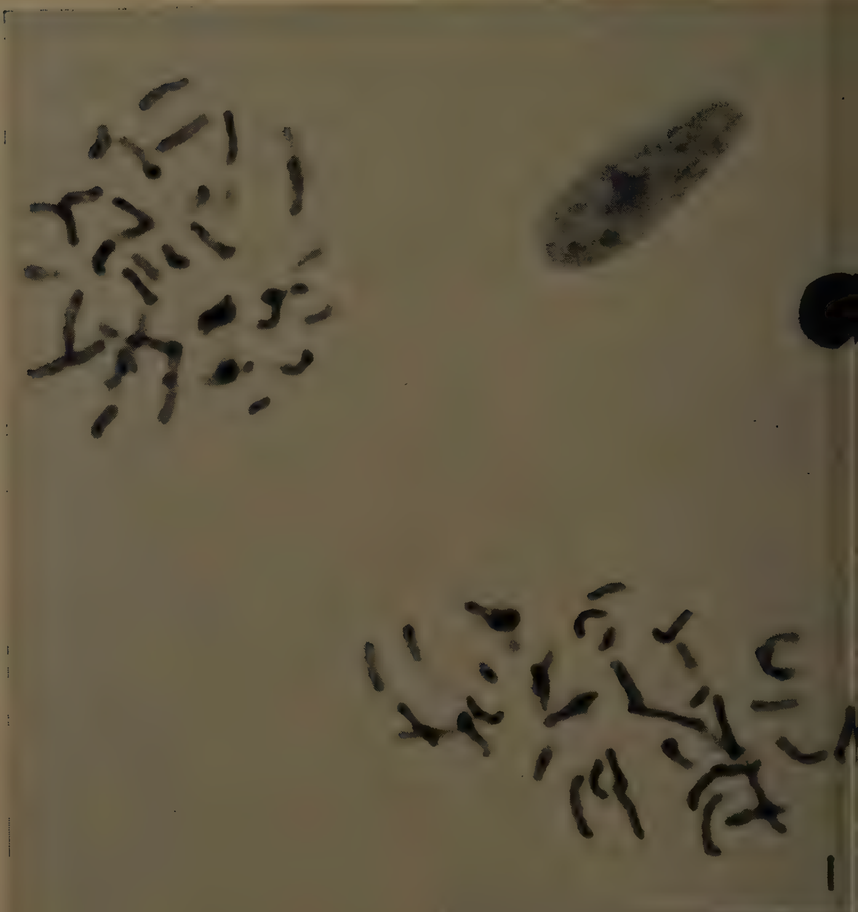
Explants Were from Mice Painted with Methylcholanthrene in Benzene (M), Painted with Benzene Alone (B), or Untreated (C).

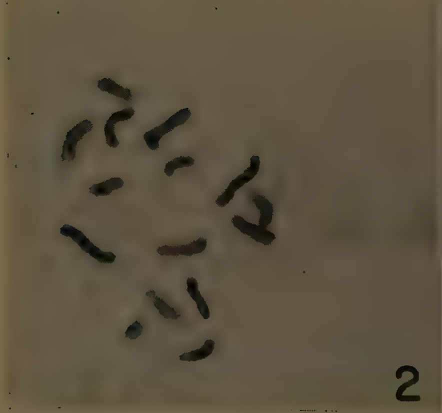
Chromosome number	Newborn			Adult		
	M	B	C	M	B	C
11	1					
12				1		
15				1		
22				1		
25				1		1
28–32				2		
33–37	2		1			
38–42	51	49	25	81	25	83
43–47	1		1	1		2
48–52						
53–57						
58–62				1		
63–67						
68–72				1		1
73–77		1	2		1	
78–82	2			7	4	4
83–87				1		
100				1		
130						1
156						1
160				1		

Frequencies of mitotic figures, according to their chromosome numbers counted in hypotonic spreads of tissue cultures of variously-treated mouse skins, are presented in TABLE 1. The data for adult skin cultures are combined results of experiments with one and five paintings. Diploid mitoses are most frequent in cultures from both newborn and adult mice, and a secondary mode of tetraploid mitoses is evident. In addition, the cultures from adult skins have a few mitotic figures with about octoploid chromosome numbers. The only effect of previous methylcholanthrene treatment to be seen here is a limited amount of aneuploidy, especially in the subdiploid range. Cultures from mice painted with benzene alone show no departure from the untreated controls.

In order to illustrate the subdiploid mitoses encountered, the two diploid mitoses of FIGURE 1, each with 40 chromosomes and photographed in a culture of skin from an untreated newborn mouse, may be compared with FIGURE 2, which has 15 chromosomes and occurred in a culture from an adult mouse painted five times with 0.3 per cent methylcholanthrene in benzene. FIGURE 3 contains only 11 chromosomes. It was in a skin culture from a newborn mouse painted once with methylcholanthrene in benzene. These mitoses with abnormally low chromosome numbers are reminiscent of the hypoploid figures described from both cancerous and normal tissues by Timonen and Therman (1950).

Other mitotic aberrations occur in our methylcholanthrene-treated material, in general confirmatory of the changes described by other authors in tissue





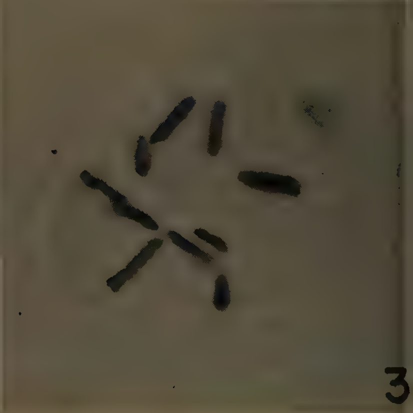


Plate I

FIGURE 1. Two mitotic figures, about late prophase, each with 40 chromosomes, in hypotonically trea culture of skin from untreated newborn mouse. Feulgen. ×2025.

FIGURE 2. Mitosis with 15 chromosomes, in hypotonically treated culture of skin from adult mouse pain 5 times with methylcholanthrene. Feulgen. ×2025.

FIGURE 3. Mitosis with 11 chromosomes in hypotonically treated culture of newborn mouse skin pain once with methylcholanthrene. Feulgen. ×2025.

cultures treated directly with carcinogens. These include, as in figure 4, the presence of undivided chromosomes near the poles in metaphase instead of on the equatorial plate. This figure is of a presumably binucleate cell in a skin culture (not exposed to hypotonicity) from a mouse painted four times with methylcholanthrene in benzene. Such a polar distribution of undivided laggard chromosomes in metaphase was regarded as especially characteristic of the effect of carcinogenic hydrocarbon in tissue culture by M. R. Lewis (1935). It has also been noted by Mauer (1938), von Moellendorff (1939), and Ludford (1953) in carcinogen-treated animal tissue cultures, and by Mottram (1934) in treated bean roots. The effect resembles the "3-group metaphases" produced by hydroquinone in certain cell types, as pointed out by Parmentier (1954).

Chromosomal bridging and fragmentation in anaphase and telophase also follow treatment with some carcinogens. Figure 5 shows a telophase with three fragments in a skin culture (not exposed to hypotonicity) from a mouse painted five times with methylcholanthrene. Similar effects were described by Mottram (1934) in bean roots treated with tar. Ludford (1953) illustrates these effects in fibroblast cultures treated with benzpyrene and methylcholanthrene, and states that they are generally considered to be the most important mitotic abnormality produced by carcinogens. Figure 6 shows a later stage in a bridged division figure from a methylcholanthrene skin culture. The chromosomal fragments at the equator, as well as the unsplit polar chromosomes, have formed micronuclei.

Our cultures of methylcholanthrene-treated skin also contained a number of mitoses such as figure 7, which seems to be two metaphase plates, of 20 chromosomes each, close together. Hearne Creech (1939) saw similar figures in mouse fibroblast cultures treated with water-soluble choleic acid compounds of carcinogenic hydrocarbons, and she interpreted them as anaphases of somatic reduction divisions. Her paper illustrated one figure of 19 plus 21 chromosomes. Figure 8 is of a similar figure with 16 plus 24 chromosomes, and we have seen a figure with 11 plus 28 chromosomes. Battaglia (1950) has interpreted this aberration to be a partial C-mitosis resulting from spindle-disturbance. The explanation would be the same that Levan and Lotfy (1949) gave for the "somatic meiosis" that Huskins (1948) thought he induced in plant material with sodium ribose nucleate; namely, that a "distributive C-mitosis" occurred. One might add that the arrangement of the chromosomes in our figures suggests that they are oriented around the centrioles as a result of a continuing centromere-centriolar attraction, despite the probable inactivation of the spindle. The figures bear a strong resemblance to the "false anaphases" produced in newt heart cultures by irradiation of spindle or cytoplasm with a narrow beam of ultraviolet (Bloom, Zirkle, and Uretz, 1955).

Figure 9 is of a tetraploid mitosis with 80 chromosomes, again tending toward an orientation in two polar groups. This was also in a culture of adult skin that had been treated once with methylcholanthrene. Hughes (1952) pointed out that hypotonic salt solution produced effects resembling C-mitosis, but in our cultures prior exposure of the skin to methylcholanthrene appeared

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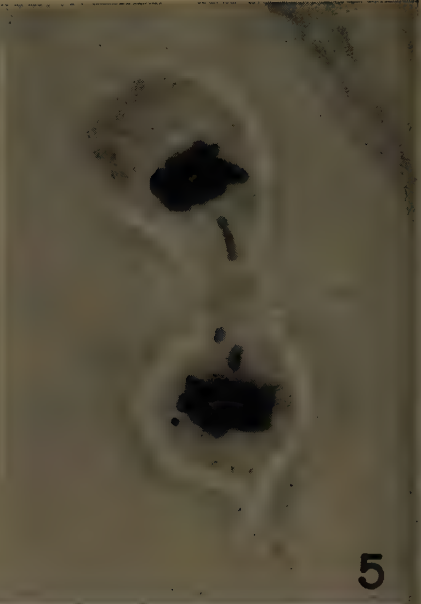

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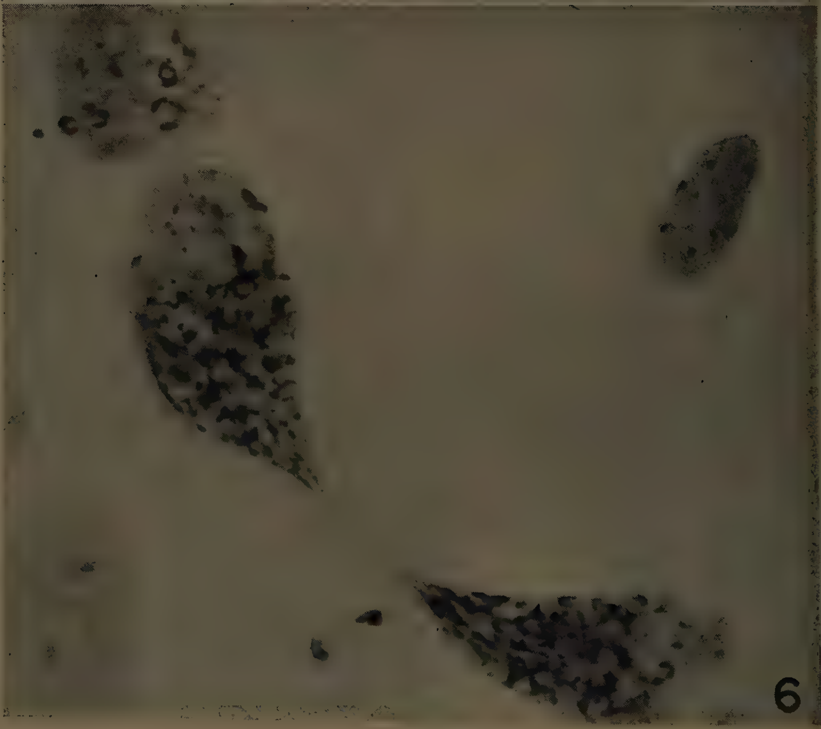

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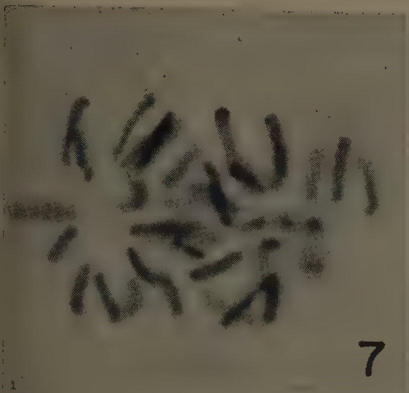
Plate II

FIGURE 4. Metaphase, probably of binucleate cell, with several laggard chromosomes toward each po
from culture in isotonic medium of adult skin painted four times with methylcholanthrene. Feulgen. ×20

FIGURE 5. Telophase with three fragment chromosomes from culture in isotonic medium of adult skin paint
five times with methylcholanthrene. Feulgen. ×2025.

FIGURE 6. Bridged late reconstruction figure with 22 micronuclei from laggard or fragment chromosom
from culture in isotonic medium of adult mouse skin painted 4 times with methylcholanthrene. Feulgen. ×13



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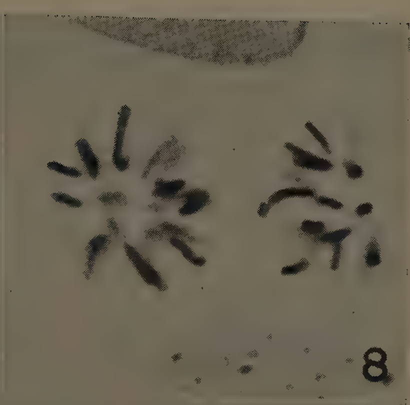

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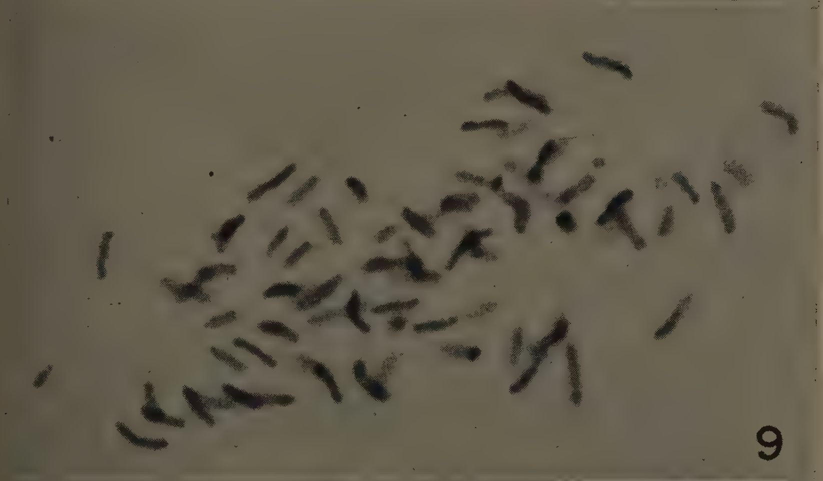

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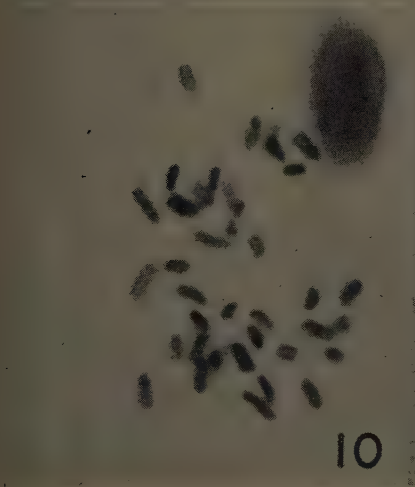

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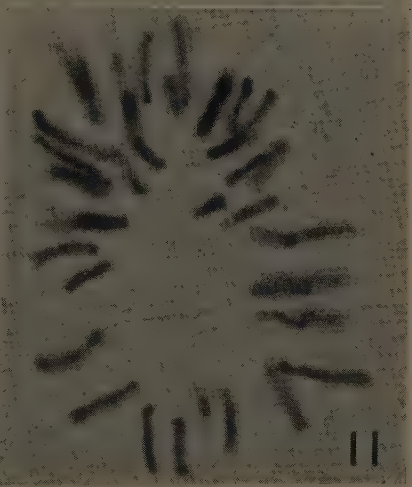

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Plate III

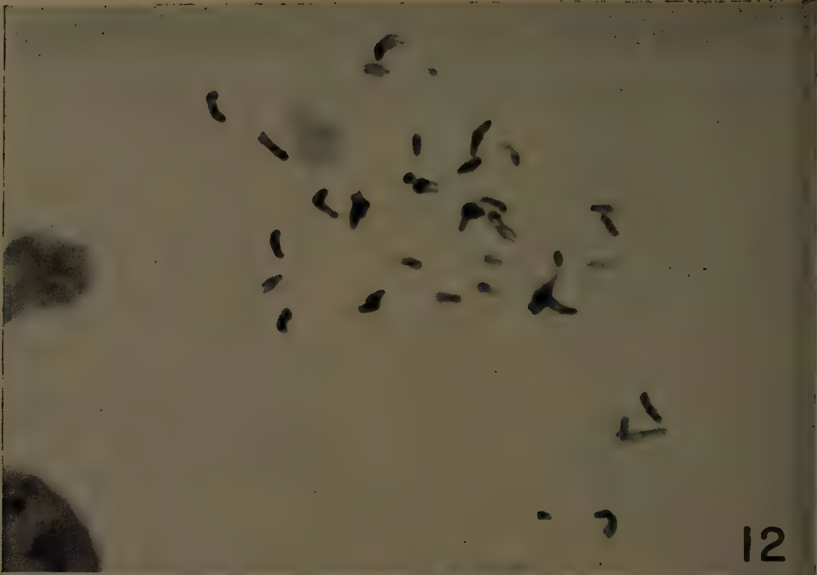
FIGURE 7. Aberrant metaphasic figure with chromosomes oriented in two groups of 20 each about the two oles of an inhibited spindle, in hypotonically treated skin culture from an adult mouse painted once with methyl- holanthrene. Feulgen. ×2025.

FIGURE 8. Two-group metaphase in culture in isotonic medium of skin of adult mouse painted four times ith methylcholantrene. Feulgen. ×2025.

FIGURE 9. Mitosis with 80 chromosomes showing tendency toward two-group orientation in culture in hypo- onic medium of skin of adult mouse painted once with methylcholanthrene. Feulgen. ×2025.

FIGURE 10. Mitosis with 40 small chromosomes in culture in hypotonic medium of skin of adult control ouse. Feulgen. ×2025.

FIGURE 11. Metaphase with 40 chromosomes in culture in hypotonic medium of adult mouse skin painted 5 imes with methylcholanthrene. Compare with FIGURES 10 and 12. Feulgen. ×2025.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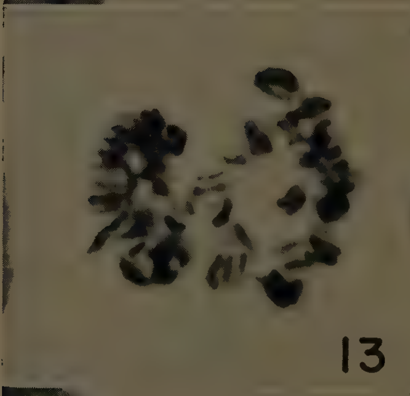
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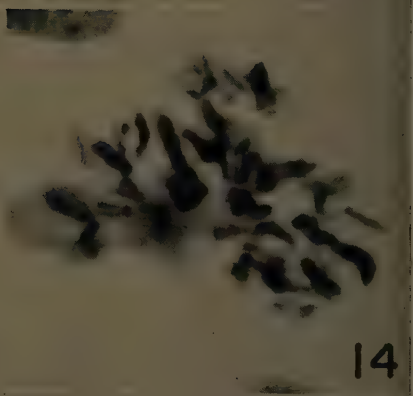
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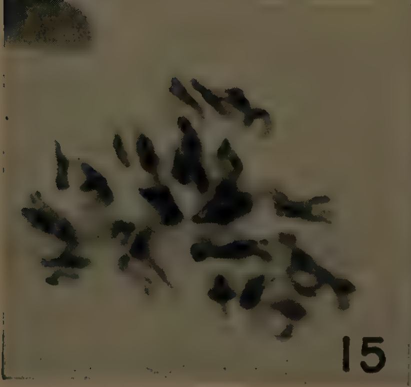
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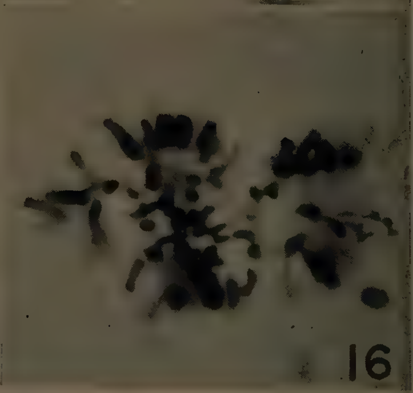
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Plate IV

Figure 12. Mitosis with 40 small chromosomes in culture in hypotonic medium of newborn mouse skin pair
once with benzene. Feulgen. ×2025.

Figure 13. Diploid metaphase with 40 diplochromosomes, each composed of 4 chromatids attached at
centromere, in culture in hypotonic medium of skin of newborn mouse painted once with methylcholanthre
This mitosis may be following an endoreduplication or a C-mitosis. Feulgen. ×2025.

Figures 14 and 15. Two focal levels of late prophase figure with 80 chromosomes, of two chromatids ea
grouped in 40 pairs, in culture in hypotonic medium of skin of newborn mouse painted once with methylchol
threne. This mitosis may be following an endomitosis, endoreduplication, or a C-mitosis. Feulgen. ×202

Figure 16. Metaphase, approximately tetraploid, with one chromosome at edge of figure greatly underc
tracted or eroded, from culture in isotonic medium of skin of adult mouse painted four times with methylchol
threne. Feulgen. ×2025.

instrumental in bringing out the bipolar orientation of the arrested metaphase chromosomes.

But we were interested in learning what tissue culture could contribute to our understanding of the apparent enlargement of chromosomes in methylcholanthrene-induced epidermal carcinogenesis. As one sees in FIGURE 9, there is little reason to suspect the presence of more than two chromatids per chromosome, although the chromosomes in the methylcholanthrene tissue cultures are often larger than the chromosomes in some mitoses of the control cultures. FIGURE 10 shows a set of 40 small chromosomes in a hypotonically-treated control culture, FIGURE 11 shows a set of 40 large chromosomes in a hypotonically-treated methylcholanthrene culture, and FIGURE 12 is of a set of 40 small chromosomes in a hypotonically-treated benzene culture. One might suspect a difference in degree of contraction of the chromosomes. Under the assumption of a constant amount of deoxyribonucleic acid per diploid set of chromosomes, one could also interpret the finding of Gopal-Ayengar and Cowdry (1947), that the amount of DNA per mg. dry weight of chromosomes isolated from mouse epidermis decreases in methylcholanthrene-induced hyperplasia, to mean that there is an increase in other materials, perhaps protein, in the isolated chromosomes. An increase in pepsin-digestible material was suggested as the basis for size increase in mouse leukemic cell chromosomes (Biesele, 1947). Mellors (1955) has discussed the possible role of nonhistone protein in accounting for the greater mass of cancer chromosomes.

Our tissue cultures provide us with another possible explanation. FIGURE 13 is a photograph of a mitosis with 40 diplochromosomes, each of which is composed of 4 chromatids still attached to one another at the centromere. The hypotonic treatment allows the multiple-chromatid nature of these chromosomes to be made out. Perhaps an acetocarmine squash of the epidermis would not permit this chromatid dissection. A similar figure is shown in FIGURE 14 although, in this, the appearance is that of somatic pairing, because each 4-chromatid group has already fallen apart into two 2-chromatid chromosomes, of which there are 80. FIGURE 15 is another focal level of the same. Each of these figures may be interpreted as a mitosis following an endoreduplication, as described for cancer cells by Levan and Hauschka (1953). The last mitosis shown, with sister-chromosomes separate but still near another, could conceivably have followed an endomitosis in mouse tumors and liver cells as described by Biesele, Poyner, and Painter (1942). On the other hand, both figures could be derived from a C-mitotic arrest in the preceding mitosis. It is of interest that these two figures were both found in cultures from methylcholanthrene-treated newborn mice, and that similar figures were not encountered in cultures of adult skin.

There is one other chromosomal abnormality to be described in these methylcholanthrene tissue cultures. This is an apparently deficient spiralization of some chromosomes but not of all in mitosis. FIGURE 16 shows a polyploid mitosis, in which one chromosome is a long-drawn-out Feulgen-positive thread with irregular thickenings or loops along its length. This severe undercontraction of one chromosome of the figure has been seen in some other mitoses of these cultures also. It was seen only in cultures of skin from the methyl-

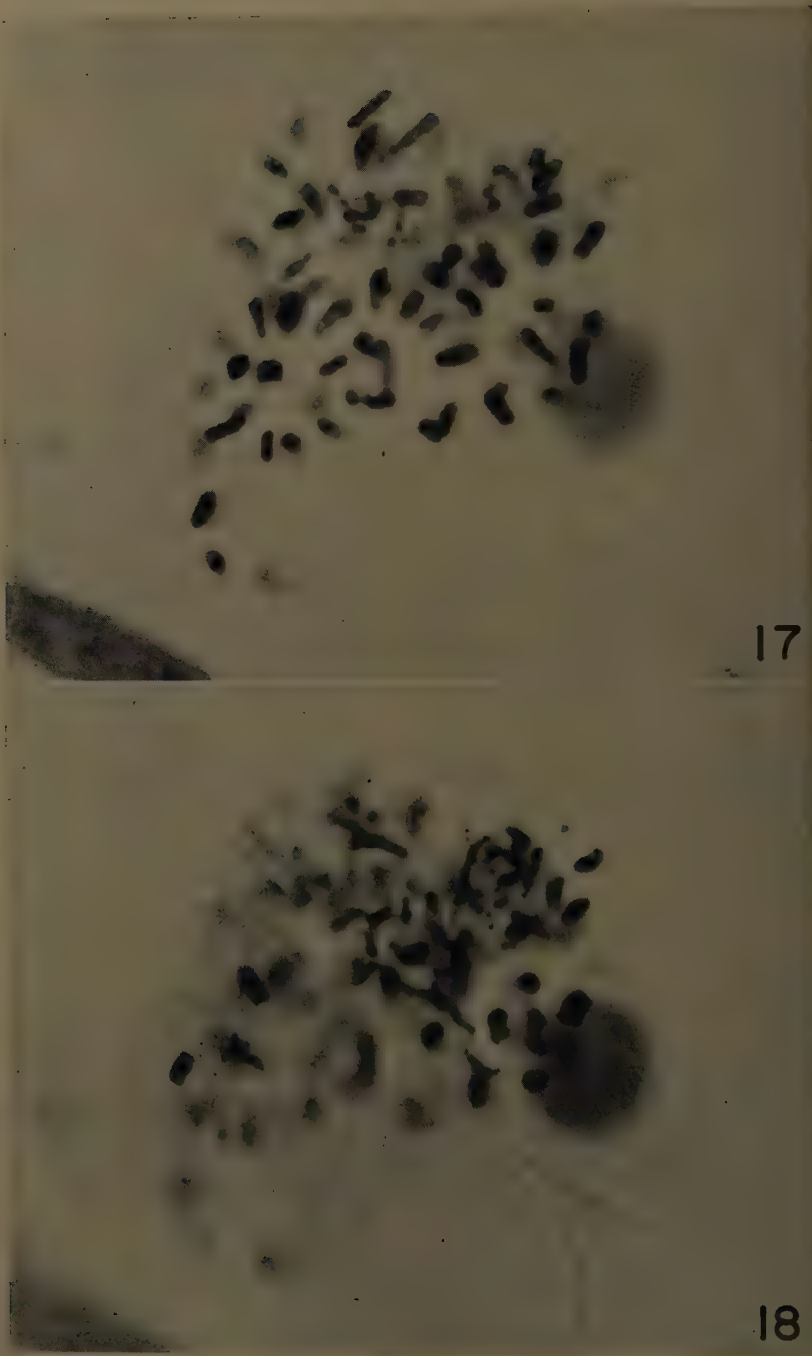

Plate V

Figures 17 and 18. Two focal levels of a tetraploid mitosis in which 20 of the 80 chromosomes are great undercontracted, from culture in hypotonic medium of skin of adult mouse painted 5 times with methylchola threne. Feulgen. ×2025.

cholanthrene-treated adult mice. Figure 17 shows the effect in a tetraploid mitosis. About 20 chromosomes of the 80 were affected. Figure 18 shows more of the underspiralized chromosomes at another focal level.

Just how these chromosomal changes may be related to the cytoplasmic effects of carcinogenic hydrocarbons discussed in the first part of this paper is an open question. It must be borne in mind, however, that the cell is an integrated system, and that effects on the cytoplasmic nucleoproteins may feed back through the nucleolus to produce indirect effects on the chromosomes.

As for aberrations in the mitotic mechanism, Ludford (1953) has discussed how various mitosis-poisoning agents, including carcinogens, may effect alterations in the distribution of water among proteins of different portions of the cell during various phases of cell division, and thus interfere with division.

Summary

(1) Known chemical and morphological changes in experimental epidermal carcinogenesis are reviewed and discussed with particular reference to the macromolecular nucleoprotein particles of cytoplasm, as a basis for an electron microscopic study.

(2) Mitotic and chromosomal changes were examined in tissue cultures of mouse skin that had been treated *in vivo* with methylcholanthrene.

(3) Several possible explanations of a chromosomal size increase in carcinogenesis are discussed, including a multiple-chromatid condition of chromosomes in mitosis following an endomitosis, endoreduplication, or C-mitosis. An increase in proteins is also a possibility.

(4) Some mitoses in cultures of methylcholanthrene-treated skin contained a minority of greatly undercontracted chromosomes.

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Part I. Perspectives in Cancer Cytology

THE CYTO-VIEWER: A NEW AUTOMATIC SCANNING MICROSCOPE

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The diagnosis of cervical carcinoma by various cytologic methods is now possible at an early stage of its development. Inaccessible regions of the body can now be reached for the collection of cellular material. The routine application of the method to all patients, regardless of symptoms, has brought to light carcinoma *in situ* lesions that, in most cases, were asymptomatic. General population screening programs carried out in Floyd County, Ga., and in Shelby County, Tenn. (Erickson*) resulted in the diagnosis of a large number of cases with unsuspected carcinoma *in situ* at a prevalence rate of from 6 to 9 per thousand.

On the basis of information collected thus far, it appears that mass screening of the entire female population may eliminate death from cervical carcinoma by its diagnosis during the curable, *in situ* phase. Such a procedure, however, would require an astronomical number of technicians, in addition to a large number of well-qualified cytopathologists. The training of a cytotechnician with a satisfactory background in histology, hematology, or bacteriology usually cannot be accomplished in less than one year. Thus, many years would be required to train an adequate number of technicians before the screening of large populations could be undertaken.

The costs of conducting such screening projects have proved prohibitive in view of the fact that a well-qualified technician cannot exceed the examination of 25 to a maximum of 35 cases per day if he is to maintain a high degree of accuracy.

In order to facilitate and accelerate the training period of technicians and to increase the daily number of specimens examined with a high degree of accuracy, a special scanning microscope, the Cyto-Viewer (FIGURE 1) has been constructed in cooperation with the engineering staff of E. Leitz, Inc., of New York, N. Y.

The Cyto-Viewer consists of a microscope with an automatic mechanical stage that can be set to cover areas of various sizes on the slide. The speed of the automatic stage is controlled by a rheostat that is connected to the variable-speed drive. The mechanical stage carries the slide horizontally back and forth until the end of the preset area of scanning is reached. At this time, the stage stops automatically. Slides may be centered for different sizes of cover glasses. Cells that appear at the edge of the screen can be centered by manual controls.

The image moves on the screen vertically either up or down, and the direc-

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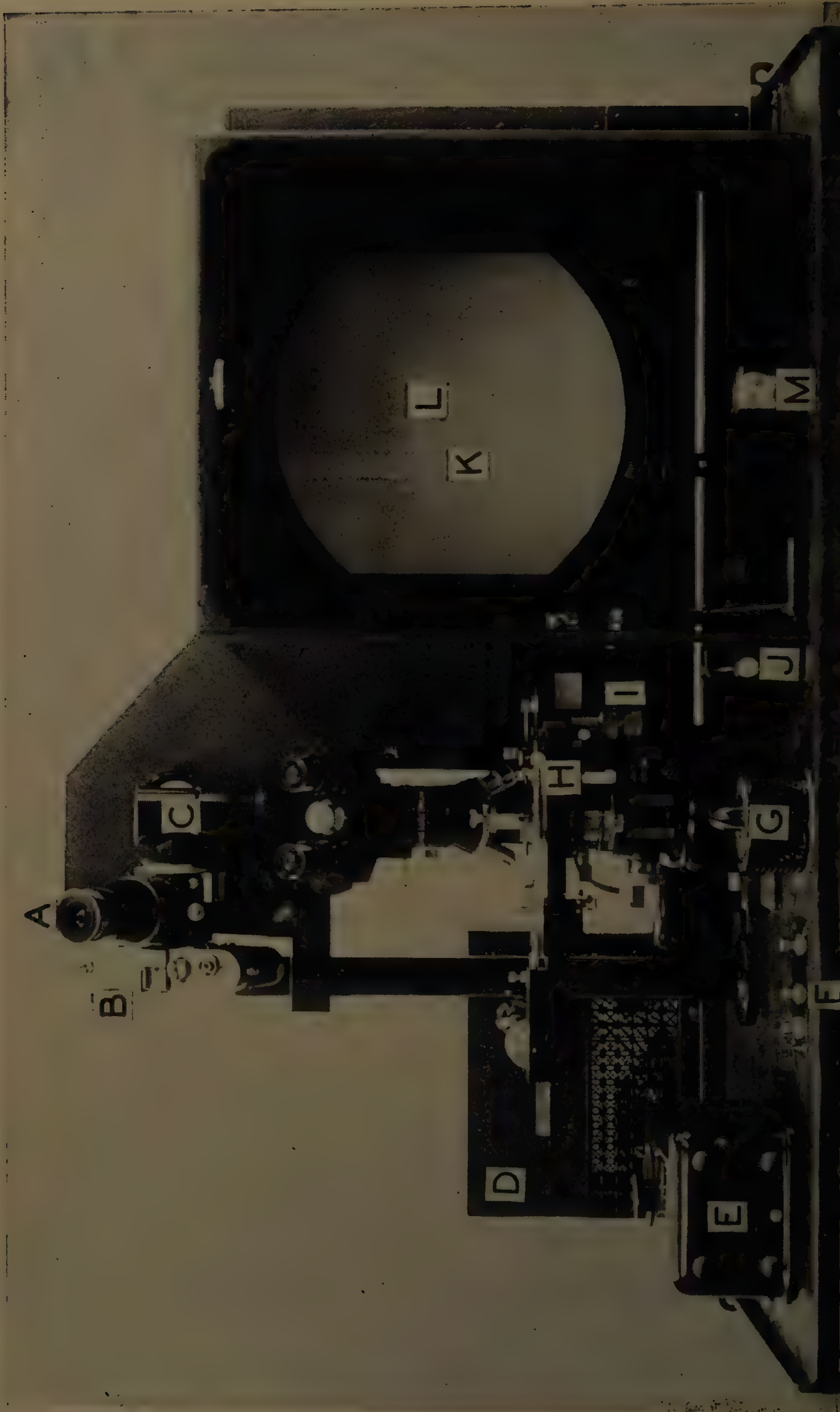
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tion can be changed at will by two manual controls. For more detailed examination of the slides, the automatic stage can be stopped instantly. Focusing of the microscope is done manually through a remote control placed in front of the screen. The microscope is equipped with objectives of 12, 24, 45, and 63 power. Different oculars may be used and the cells, therefore, may be examined on the screen under different magnifications.

The stage is equipped with a micrometer consisting of a cross line that can be moved horizontally to any part of the screen for detailed measurements of cells by turning a micrometer screw. With a 12-power objective and 10× ocular, one mm. on the micrometer scale is equivalent to 2.5 microns.

The microscope also has a 35-mm. camera attachment. The reflex focusing magnifier of the camera is synchronized with the screen, and any image on the screen may be photographed by simply turning a lever and manipulating the dual cable release. Although the image is generally in focus on the camera attachment simultaneously with the screen, the microscope, if necessary, may be brought to sharper focus by manipulating the control placed in front of the screen.

The Cyto-Viewer is of considerable value for teaching, since it eliminates the manipulation of individual microscopes and permits the demonstration of cell structures to a sizable group of students. A greater number of specimens can therefore be examined simultaneously by a large group. The examination of slides on the screen is accomplished without eye strain while cellular details are well defined. The binocular attachment of the microscope has been incorporated in the experimental model, but appears to be unnecessary, since it is rarely used.

Cellular abnormalities are clearly visible on the screen to such an extent that an endocervical smear can be examined in 2 minutes and 10 seconds with 100 per cent accuracy. The microscope has been tested for teaching purposes and for the examination of specimens, which included 100 cases of known cancer.

It is suggested that the use of the Cyto-Viewer may allow the screening of the general population for cervical carcinoma as for cancer in other sites of the body at a considerably lower cost than hitherto possible, and at an earlier date than anticipated.

FIGURE 1. The Cyto-Viewer. (a) Reflex housing with focusing magnifier. (b) 35-mm. Leica camera. (c) Lever to direct image from screen to camera. (d) Variable speed drive for mechanical stage. (e) Transformer for microscope lamp. (f) Control knobs for stop and go, also for upward or downward movement of image on screen. (g) Rheostat connected to variable speed drive for regulating rate of movement of mechanical stage. (h) Mechanical stage on microscope, mechanically connected to variable speed drive. (i) Manual controls for mechanical stage. (j) Micrometer screw for measuring images on screen. (k) Cross-line connected with micrometer. (l) Transparent screen. (m) Focusing adjustment by remote control to fine adjustment on microscope.

IDENTIFICATION OF CELLS IN EXUDATES AND EFFUSATES*

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The identification of cells in exudates and effusates presents a problem tha differs in many respects from that posed by the examination of other bod fluids for cancer cells. In the first place, the material is very copious, ofte measurable in liters rather than mils, and the cells suspended in it are, of necessity, widely dispersed. In a vaginal smear, or in one from sputum or gastri washings, the cells are mixed with mucus and, often, the smear may be prepared without doing anything more than transferring this mucus to a glas slide and spreading it thin. Copious bronchial washings or large specimen of urine must be centrifugated to concentrate their cellular contents but, eve so, they are not to be compared with the voluminous accumulations of serou fluid in, let us say, ascites from hepatic cirrhosis. After centrifugating a larg amount of such fluid, correspondingly large numbers of cells are thrown dow in the sediment and most of these are, if not normal, at least nonneoplasti constituents of the body cavity involved. Thus, in the examination of smear of such sediment, one must be able to distinguish between these habitua denizens of the thoracic or peritoneal cavities and the sinister elements tha wander into them from malignant tumors. This takes a good deal of practice as these nonneoplastic cells may undergo considerable metaplastic change an take on an appearance disturbingly similar to that of cancerous elements.

The next problem to be considered is a practical and clinical one: havin identified cancer cells in a fluid, what can be done about this in the way o taking curative measures? The answer is: "Nothing." By the time that body cavity is flooded with fluid containing cancerous elements, it is too lat to think of operating, and the outlook for therapeutic use of radioactive agent such as the X ray, radium, or radioactive isotopes is not at all encouraging All that can be expected of the cytologist is a decision as to whether or no cells of malignant tumors are, or are not present in the exudate or effusate and possibly, a hint as to the site of origin of tumor and its histologic type. Thi may sound pessimistic, but it should not. After all, the physician is intereste in knowing where he stands, and whether or not he is dealing with an inflammatory or mechanical disturbance or, as he probably suspects, with a case o generalized carcinomatosis. His treatment of the patient will depend upo the report of the cytologist. If this report should be encouraging to the exten that there is no definite evidence of malignant growth, not only will the patient's physician be vastly relieved, but needless diagnostic procedures suc as biopsies and possibly exploratory laparotomy will be avoided as impracticable and useless.

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* This article is based largely on an investigation supported by a research grant (C-2401-PATH.) from th National Cancer Institute of Health, United States Public Health Service, Bethesda, Md.

Thus, the examination of serous effusates and exudates is of minor value in the early detection of incipient cancer. The only condition in which it might conceivably be of use in that respect would be in that of the rare primary tumor of mesothelial origin that may occasionally be encountered in the pleura or peritoneum, or in that of malignant synovial tumors within joints. Practical experience teaches us that these forms of growths—malignant mesotheliomas and synoviomas—are seldom if ever recognized as such by the cytologist.

Methods of Examination

There are two ways in which cells in exudates or effusates of this sort may be recovered and examined microscopically: the specimen must first be centrifugated, and then the sediment may be spread out over glass slides in the manner of the familiar Papanicolaou smear; or it may be fixed while still in the centrifuge tube and the resulting button removed and embedded in paraffin, from which conventional sections are then cut. For fixation, ether and alcohol in equal parts, or some other fixing fluid such as formalin or Bouin's solution may be employed. If the former be used, the material will be suitably fixed either for smears or for paraffin blocks. In preparing smears, it is usual to make them from the sediment and then to discard any of this that has not been utilized. It is far better to take as much as is necessary for the preparation of smears and then to save and fix the rest of it in the tube and "block" it, than it is to pour it down the drain. We shall see later that smears and blocks both have their advantages, particularly in connection with the examination of sediments of serous fluids. This being so, it is well to avail oneself of these advantages and not to neglect any possible means of making the difficult decisions that are often presented by this branch of exfoliative cytology. There is another practical reason for making cell blocks from a portion of the specimens submitted, and that is the matter of future specimens, or replacement of broken or lost ones. A smear is unique. It cannot be duplicated, nor can it be replaced, and the cytologist is usually unwilling to send it away for examination by other physicians for fear that it will be broken or mislaid. A cell block will furnish many sections, and new ones may be cut and stained at any time. Furthermore, one section will be very much like the succeeding one, so that slides for circulation will present a series of essentially similar pictures. The cytologist will have no hesitation in having them prepared and mailing them about the country.

Interpretation of Smears

The interpretation and diagnosis of smears of sediments of serous fluids is somewhat different from that of smears from vaginal, pulmonary, or gastric secretions. In these, one deals chiefly with epithelial elements, whether normal or abnormal and neoplastic. In the case of the exudates and effusates, however, the normal standard is one that comprises much fluid and few cells. Furthermore, these cells are limited to a few exfoliated mesothelial elements from the serous membranes, and to leucocytes, histiocytes and, sometimes, erythrocytes. Of these, the mesothelial cells are the most troublesome when it comes to determining the presence or absence of cancer.

Mesothelial cells. These cells are somewhat larger than histiocytes, and they have a finely granular, acidophilic cytoplasm with a "ground glass" appearance. Around the periphery of some of them, there is a circle of tiny vacuoles that lie just within the cell membrane and give to the cell something of the appearance of a circular saw. Usually there are only a few such cells in a serous exudate or effusate, but they may become multitudinous under certain conditions. They generally lie discretely separated in a smear, but they may be grouped into rosettes or pseudoacini that closely imitate bits of tumor. The former are probably the result of the proliferation of cells in the excellent culture medium afforded by serous fluid. The latter, most likely, are chains of cells that contract into a circular arrangement. Another troublesome characteristic is their propensity to form concentric groups of elements that are closely integrated and have the appearance of the epithelial pearls in epidermoid cancer. Individually, these cells may be vacuolated and thus ape the signet-ring cells of mucous cancer. Not infrequently, they show multiple nuclei. All these factors make it very easy for the beginner in cytology to make false positive diagnoses of cancer when cancer, in reality, is not present. One other feature should be mentioned. Mesothelial cells may actively engage in phagocytosis, particularly the ingestion of degenerated polymorphonuclear leucocytes in purulent exudates, such as those noted in empyema or peritonitis.

In the course of a review of 2029 specimens of exudates and effusates submitted to our laboratory during the past eight years, a job that is not yet finished, it was found that mesothelial cells were almost always at fault when false positive diagnoses had been made. Even practiced cytologists sometimes mistook them for cancer cells. In this series, examining smears without any knowledge of the history of the patients from which they were taken, I made 64 false positive diagnoses, or 10 per cent of all my positive ones. The laboratory, with knowledge of the cases, made 39, or 7 per cent of all their positives. By reviewing the smears concerned and by making a careful study of the characteristics of their cellular elements, this time with cognizance of clinical data and of the source of the fluid, these figures were reduced to 33 for me and 20 for the laboratory, or 5.5 and 4.5 per cent respectively.

It seems that careful attention to the cytologic characteristics of the nuclei is the best corrective of false impressions. In mesothelial cells, the nuclei are precisely stained, their nucleoli are relatively small and inconspicuous, and their karyosomes are delicate and not clumped into masses. In 1937, I published a report[1] dealing with this subject in connection with the examination of sections of cell blocks, and stated that the ratio of the nucleolar and nuclear diameters, obtained by dividing the former by the latter, as recommended by Quensel,[2, 3] gave important assistance in coming to a conclusion. Cells showing a nucleolar-nuclear ratio lying between 0.15 and 0.20 were almost invariably nonneoplastic, while those with ratios between 0.25 and 0.40 were as often neoplastic. The statistical region between 0.20 and 0.25 is one of doubt and uncertainty. This idea was so amply confirmed by the review of false positive diagnoses in the present investigation that there is no reason for changing my original opinion on this lead. After measuring a few hundred nuclei and nucleoli, one becomes so practiced that actual measurement is no longer necessary and may be substituted by estimation.

Thus, it is the normal elements in these smears that give us trouble. Before attempting to qualify as an expert on such matters, it is necessary that the cytologist become thoroughly familiar with the mesothelial cell and its numerous foibles and disguises. Of two excellent articles by Luse and Reagen, the first[4] is devoted to this very subject and cannot be too highly recommended; the second[5] deals with neoplastic cells and affords a very satisfactory contrasting idea of the diagnostic features involved.

Histiocytes. As usual in cytologic specimens, these versatile cells play a very important rôle in a variety of chronic inflammatory conditions. In tuberculosis, they may readily lead one astray, as they become typically "epithelioid" and may assume fairly metaplastic and neoplastic characteristics. Again, one must focus on the nuclear features. It would seem probable that the Langhans giant cells would be an important road sign in such cases, but these cells are found in greater numbers in other conditions as well as in tuberculous infections. Luse and Reagan have found that, in cirrhosis and in congestive heart failure, they occur in about one third of the specimens as against one quarter of those representing tuberculous inflammation.

Positive Diagnoses

Thus far we have been considering the difficulties encountered in the diagnosis of neoplastic cells in smears or sections of cell blocks. These were purposely emphasized because they are more troublesome in this type of material than in the case of other types of specimens. There are, after all, the 90 per cent or more of successfully diagnosed smears to be considered with considerable satisfaction. In the routine practice of cytology, it is usual to receive more specimens from one body cavity than from another. In the series now undergoing study, there are 1301 pleural, 700 peritoneal, 28 pericardial, 17 articular, and 3 hydrocele fluids. This is 63.5, 34.2, 1.4, 0.8, and 0.1 per cent respectively. Pleural fluids far exceed the other types, their nearest competitor being the peritoneal specimens which, even so, amount to only a little more than one half of the pleural fluids. Neoplastic cells are very apt to be found in the pleural and peritoneal fluids. They are seldom observed in pericardial fluids, and were not observed in our articular or hydrocele specimens.

Cells from Malignant Growths

Malignant tumors metastasize to the membranes lining these cavities, seed themselves out over their surfaces as small plaques of neoplastic tissue, and proceed to exfoliate cells into the fluid. It should be remembered that this s an excellent culture medium and is kept constantly at body temperature, so that the exfoliated cells not only can, but do multiply and form spherical aggregations that float free in the fluid. These "proliferation spheres" may often be of assistance in making a diagnosis when they are found in their entirety in smears or sections of sediment.

In recognizing cells from malignant growths, we must bear in mind the fe tures that Papanicolaou has emphasized as a basis for postulating criteria a diagnosis of cancer. These comprise: inequality in size and shape of t cells (anisocytosis); of their nuclei (anisokaryosis); overstaining of the kary plasm (hyperchromasia) resulting from a coarsening and massing of nucle material and thickening of the nuclear membrane; and abnormal mito figures. Certain tumors present other features that can be utilized; for e ample, the orangeophilia of the cytoplasm of epidermoid cells in squamo carcinoma. The mere presence of mitosis is of relatively little importance, mesothelial cells and histiocytes are wont to multiply very vigorously in the fluids. The mitoses that point tumor-wards should be of the coarse, deforme and abnormal type before they can be taken into consideration. Laggi chromosomes, Y-shaped arrangement of chromosomes and spindles, unequ numbers of chromosomes at the poles in metaphase, and other such distortio usually appear only in neoplastic cells.

Having these criteria in mind, it will be seen that the recognition of ce from malignant tumors is usually relatively easy. Many smears show hord of abnormal cells of all sizes and shapes, and of so bizarre an appearance th they could not be accepted as representing anything nonneoplastic or norma These are the smears concerning which there is no disagreement between o servers. It should be stressed, however, that the criteria just mentioned a seldom if ever valid if taken singly. Several of them should be noted befo one comes to a decision as to the presence of cancer. There is, natural enough, a borderline, or no man's land, in which decision may be difficult a largely a matter of personal opinion, but this is consolingly limited and sma

It is in this zone that the false positive diagnoses are made. For that reaso it is better for the inexperienced cytologist to limit his positive diagnoses those cases in which evidence is outspoken and to issue guarded reports those in which there is doubt. Such reports automatically imply that anoth specimen would be desirable. Such repeat specimens often show unmista able evidence of malignant growth being present. As to negative diagnose there is little to be said. If a given specimen is found to be negative for canc cells, it is no proof that cancer is absent. It means merely that, if it be pre ent, it is not exfoliating any cells into the fluid.

Recognition of Types of Cancer

Having received a positive report, the physician is very apt to wish to kno what type of cancer is present and where it originated. In many cases the will be the clinical history of a cancer having been removed from somewhe in the body, and the likelihood exists that a malignant tumor exfoliating in serous fluid will represent a recurrence, or the stepping-up of the incidence metastatic foci from some residuum of that growth. It is possible, howeve to recognize with practical accuracy the type and site of origin in the case several forms of cancer. In an article published in 1954,[6] I reported the r sults of an investigation along these lines in which it was found that, while th over-all accuracy of diagnosis in a series of 219 specimens was not very promi ng, still, it did attain practical proportions when limited to five of the tumors hat comprised the bulk of the material. There were 57 instances of mamnary carcinoma, diagnosed as such with an accuracy of 70 per cent; 49 of ronchogenic carcinoma with a diagnostic accuracy of 77 per cent; 36 of ovarian arcinoma in which accuracy fell to only 58 per cent; 17 of gastric carcinoma n which the percentage was 71, and, finally, 18 tumors grouped as "lymphoma" nd including 3 cases of Hodgkin's disease. In this latter group the accuracy mounted to 66.7 per cent.

In making these diagnoses, the clinical history of the patient was again unnown, so that the examination was truly objective. In the investigation hat is still under way, no attempt was made to diagnose type and site of rigin accurately. Each specimen was considered chiefly from the standpoint f determining the presence or absence of cancer cells, and comparatively little ime was devoted to a more careful cytologic study of each specimen. Instead f studying each smear meticulously, the areas dotted for identification by he screener were checked over, and a decision as to type was made rather apidly. In many instances (30 per cent or so of positive diagnoses), no guess vas hazarded as to these points, the specimens being entered in the "no diagosis" column. The results of this cursory examination were decidedly inerior to those obtained in the earlier investigation. There were now 66 mamnary carcinomas diagnosed as such with an accuracy of 47 instead of 70 per ent; 85 bronchogenic carcinomas with an accuracy of 42 instead of 77 per ent; 54 ovarian carcinomas with only 22 per cent accuracy instead of 58; 32 "lymphomas" with an accuracy of 59.5 per cent rather than 66.7; 24 gastric arcinomas with an accuracy of 29 per cent instead of 71; and, finally, 65 misellaneous carcinomas and sarcomas diagnosed with a score of 30.0 per cent. The figures reflect, in a measure, the relative degree of difficulty in making iagnoses in each group. The lymphomas presented the least, while the varian carcinomas offered the most.

What was the reason for this decidedly poorer showing? It is obvious that n order to arrive at approximately accurate diagnoses of types and sites of rigin of such tumors, more time than usual must be devoted to studying the eoplastic cells. Another point that was brought out by the second investiation was that there were many cases left undiagnosed in this respect that ight have been correctly evaluated after a little longer study. This might ndicate that it pays to try to reach a conclusion. The reason why lymphoma nd mammary carcinoma yielded the best results lies in the fact that the apearance of the former is very characteristic, and that mistakes occur only hen there is a copious exudate of lymphocytes that might be misinterpreted s representing neoplastic lymphoid cells. In the case of mammary carcinoma, e presence of concentrically-arranged nests of cells, like those of epithelial earls, proved to be of definite assistance. Bronchogenic carcinoma, on the ther hand, offered little in the way of histologic or cytologic peculiarities that uld be readily utilized in a rapid evaluation. While the presence of double ontour in the cellular membrane of cells of ovarian carcinoma was distinctly elpful, it was not a monopoly of these cells. The wretched showing in the recognition of ovarian carcinoma depends upon its great variety, not only type, but of morphology. Such a multiplicity of special malignant tumors attributable to the ovary that this is not surprising.

The space at my disposal has limited this presentation to a simple outli of the essential features of this branch of cytology. There are many poin that could be discussed at greater length, and they will be when the paper no in the process of construction is published.

Summary

(1) The examination of the cells in serous exudates and effusates does n offer much promise in connection with the early diagnosis of incipient cance which is invariably anything but incipient by the time its cells have exfoliate into such fluids.

(2) Mesothelial elements present the chief stumbling-block in the interpr tation of smears or cell-block sections of the sediments of these fluids, as the may imitate the appearance of cancer cells under several circumstances.

(3) Methods of examination have been discussed, contrasting the relativ advantages of smears and sections.

(4) The appearance of neoplastic and nonneoplastic cells has been describe and contrasted.

(5) Criteria for a diagnosis of the presence of malignant tumors have bee stated and discussed.

(6) The possibility of recognizing types and sites of origin of tumors throug the appearance of their cells has been mentioned. It has been found that th requires more time and study than is usually devoted to a determination onl of the presence or absence of evidence of malignant tumor.

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A HISTOCYTOLOGIC AND ELECTRON MICROSCOPIC STUDY OF EFFUSIONS ASSOCIATED WITH MALIGNANT DISEASE*

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The morphologic characteristics of cellular forms in serous effusions has been of continued interest during the past 80 years. Visualization of these cells has been obtained by various stains on either direct smears or sections of paraffin-embedded cell blocks. The recently available techniques of phase and electron microscopy offer additional information concerning the fine structure in the cellular constituents of effusions.

Materials and Methods

This is a study of 1964 fluid specimens obtained from the pleural, peritoneal, and pericardial cavities. Of these, 821 were from patients with malignant neoplasms. The remaining 1143 specimens were considered of nonneoplastic origin. The cause of the effusion was known for 554 of the 1143 specimens, while in 589 the etiology was not definitely established. In none of these 589 fluids was there evidence that a malignant tumor contributed to the accumulation of the serous fluid.

The 821 effusions associated with neoplasms were from 427 patients, in all of whom there was definite evidence of a malignant tumor. It could not be proved in all cases, however, that the tumor was the direct cause of the effusion.

A majority of the fluid specimens were examined as paraffin-embedded cell blocks, sectioned and stained with hematoxylin and eosin. This method of preparation has been described in detail.[7] Recently, an easier and more satisfactory manner of handling the fluid specimens has been used. To each 100 cc. of the fluid was added 10 cc. of 40 per cent formaldehyde. This was allowed to stand at room temperature for an hour, and then was centrifuged. To the cell mass obtained by centrifugation was added approximately 2 cc. of melted agar. This was gently interspersed so as to hold the cells together, chilled briefly in order to solidify the agar, removed from the centrifuge tube, and sectioned into pieces of a convenient size. The agar then held the cells together during dehydration and paraffin embedding. The background staining of the agar was slightly acidophilic, but in no way did it interfere with examination of the formed elements. The avoidance of alcohol prevented the precipitation of protein and dilution of the cellular constituents.

Nine of the effusions were studied by phase microscopy and electron microscopy, as well as by the cell-block technique. A portion of these fluids was ixed with osmic acid as soon as possible following removal of the effusion from the patient. The fixative used was a 2 per cent solution of bichromate-buffered osmium tetroxide as recommended by Dalton.[2] One part of buffered-osmiu tetroxide was added to three parts of fluid and allowed to stand at room tem perature for 30 minutes. This mixture was centrifuged at low speed for minutes, the supernatant decanted, and cooled but still liquid agar adde The solidified agar containing the cells was sectioned into small pieces, de hydrated in graded alcohols, and embedded in a three-to-one mixture of buty and methyl methacrylate. The methacrylate was polymerized at 45°C., th process being catalyzed by benzoyl peroxide. Thin sections were cut wit glass knives[6] on a modified Minot rotary microtome[3] and examined in a RC electron microscope (model EMU 2E) without removing the plastic. Adjacen thicker sections were used for phase microscopy. Some of the centrifuge cells were examined by the phase microscope as direct wet smears after bot formalin and osmic fixation.

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* This investigation was supported, in part, by grants B-425 and C-2174 from the National Institutes of Health, United States Public Health Service, Bethesda, Md.

† Fellow of the American Cancer Society, administered by the Committee on Growth, National Research Council, Washington, D. C.

Nonneoplastic Cellular Elements

The various cellular components observed in effusions not associated wit malignant neoplasm are of importance. The most commonly observed form are mesothelial cells, lymphocytes, mononuclear cells, neutrophils, eosinophil and erythrocytes. Most important were the mesothelial cells and lymphocytes. Rarely were the other cellular forms difficult to distinguish from malignant tumor cells.

The mesothelial cells were often isolated. They were ovoid or round an presented a moderately abundant finely granular acidophilic cytoplasm. Th nucleus was round or oval, sometimes indented and, not infrequently, eccentri in position. A small round nucleolus was usually present.

In degeneration, the cytoplasm of the mesothelial cell underwent alteratio forming either multiple small vacuoles or a single large vacuole (FIGURES 1 16, and 18). The single large vacuole displaced the nucleus, compressed it and gave a pseudosignet-ring appearance to the cell. Phagocytosis of erythrocytes by mesothelial cells was observed occasionally.

Giant multinucleated mesothelial forms were present in some fluids. Clump of mesothelial cells were relatively common and, usually, were easily distinguished from neoplastic cells. In occasional effusions, however, the mesothelial cells were present in an acinar arrangement. In a rare fluid, the acinar like forms were numerous and distinguished from neoplastic cells with difficult if at all (FIGURE 2). These cells were usually arranged radially about a clea central space. The nuclei tended to be of a uniform size and shape, althoug they occasionally showed a prominent, coarsely granular chromatin. Th acinarlike arrangement was most often seen in cases of chronic effusion associated with congestive failure or in cirrhosis. Occasionally, however, it wa seen with one of the lymphomas.

Lymphocytes were common in effusions, but rarely were they the predominant element except in effusions associated with tuberculosis or with lymphoma Occasionally, in a tuberculous effusion, lymphocytes were so numerous tha a diagnosis of lymphatic leukemia or lymphosarcoma had to be considered Usually, the presence of relatively few mitotic figures, a majority of smal

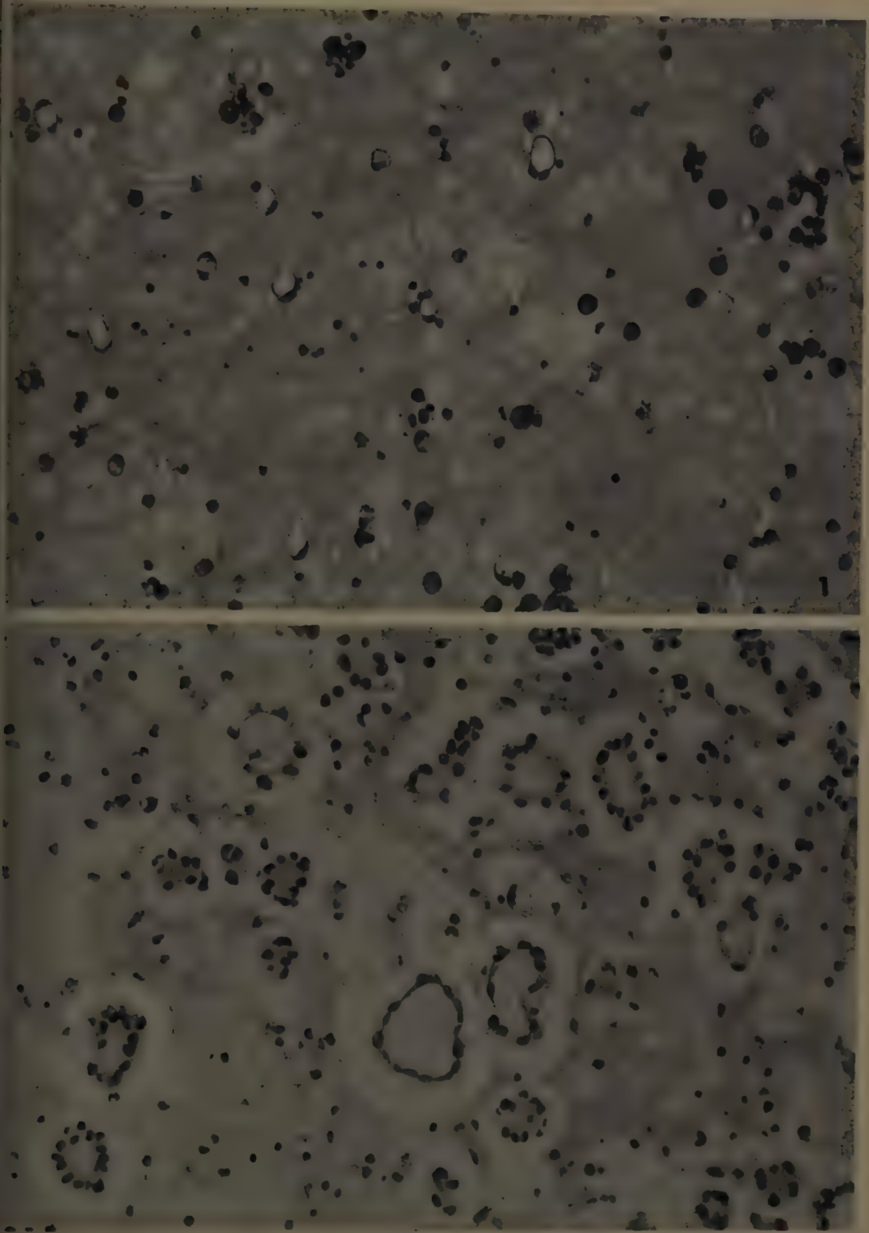


Plate I

FIGURE 1. Pleural effusion in congestive failure. Note clumps of mesothelial cells at upper left. Numerous acuolated mesothelial cells (pseudosignet ring forms) are present. Compare vacuolated cells with electron icrograph, FIGURE 18. ×215.

FIGURE 2. Pericardial effusion showing proliferation of mesothelial cells with numerous acinic forms and oc asional aggregates. This type of mesothelial alteration is often confused with malignant tumor. ×215.

mature forms, and an intermixture of mesothelial cells will distinguish th
tuberculous effusion from that due to lymphoma.

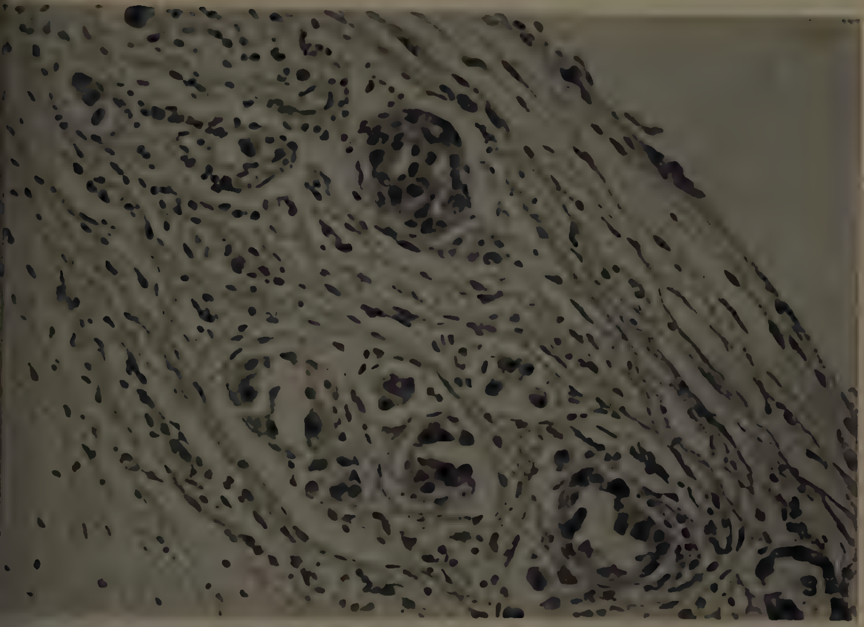
Neoplastic Effusions

This study reports the incidence of malignant tumor cells in 821 effusion
obtained from 427 patients with malignant neoplasms. This is a continuatio
of the work previously reported by us.[7] The frequency of neoplastic cells i
the entire series and in the various types of lesions is shown in Table 1 of tha
work. Although all of these patients had malignant tumors, it is not possib
to state that, in all cases, the effusion was due to metastatic tumor in a serou
cavity. In some cases, especially among the lymphomas, the effusions ma
have been the result of venous obstruction. In other cases, there was a prom
nent desmoplastic response in the serosal metastases (FIGURE 3). In such le
sions, the neoplastic cells were covered by fibrous connective tissue that ma
have impeded exfoliation of the tumor cells. Although the section shown i
FIGURE 3 is a metastasis from an esophageal carcinoma, a similar picture
often seen in metastatic lesions secondary to carcinoma of the breast.

Malignant tumor cells, mesothelial cells, lymphocytes, neutrophils, eosino
phils, erythrocytes, megakaryocytes, and various other cellular forms we
seen in the effusions associated with neoplasms. The cancer cells were var
ously arranged, some occurring as isolated cells while othere were clumped
arranged in acinarlike or papillary forms. In some fluids, actual fragmen
of tumor were present. In general, tumor cells in sections of cell blocks
fluids were larger than the nonneoplastic elements. In sections in which th
tumor cells were measured by a micrometer, they varied from about 10 micron
to over 50 microns in diameter. Often the cytoplasmic boundary of the ce
was indefinite. The cytoplasm of the cancer cells was acidophilic, granula
and, frequently, it contained mucicarmine positive vacuoles of variable siz
In many cells, these vacuoles distorted the nucleus, pushing it to one side
the cell. In some tumor cells, more than one nucleus was present while, i
others, large and bizarre nuclear forms occurred. Often the nuclear membran
was irregular, but this was not invariable. The nuclear chromatin was
varying coarseness and density, an occasional nucleus being opaque. Nucleo
were prominent.

Bronchogenic Carcinoma

Cancer cells were rarely identified in fluids associated with small cell
squamous cell bronchogenic carcinoma. When present in effusions derive
from small cell tumors, the cells were infrequent, small, and had a scant cyto
plasm. Among those fluids associated with squamous cell carcinomas i
which neoplastic cells were present in the cell blocks, it was rare to find a fo
mation suggestive of their squamous origin. In contrast, pulmonary aden
carcinoma was often associated with large numbers of exfoliated neoplast
cells. In some effusions, the cells formed large acinar formations and clump
Mucicarmine positive material was present in the vacuoles. A cellular pi
ture that was seen with no other group of neoplasms was frequently associate



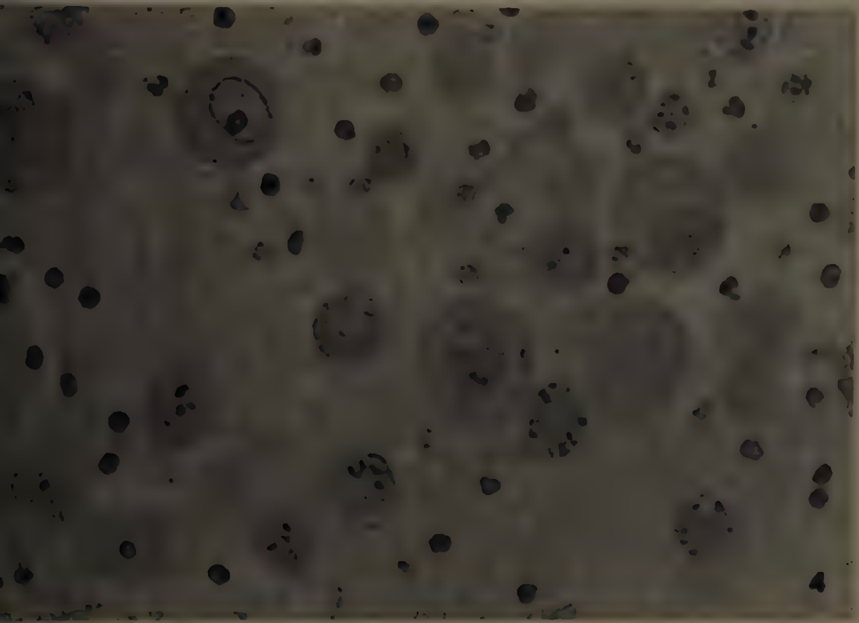


Plate II

Figure 3 Biopsy of pleura showing foci of metastatic esophageal carcinoma with fibrous connective tissue vering the nests of neoplastic cells A narrow band of intact mesothelial cells can be seen on the pleural sur- ce at the right. ×215.

Figure 4 Adenocarcinoma of lung Pleural effusion. Large isolated neoplastic cells with vacuolated toplasm, large nuclei, and macronucleoli. ×600.

with adenocarcinoma of the lung. In these effusions, there were numer large isolated neoplastic cells with bizarre nuclei, macronucleoli, and vacu lated cytoplasm (FIGURE 4). Mitotic figures were frequent. Occasion acinous forms occurred but, in some of the fluids, the large isolated cells we the only neoplastic elements present. In our experience, this cellular pictu has always been associated with adenocarcinoma of the lung.

Gastrointestinal Neoplasms

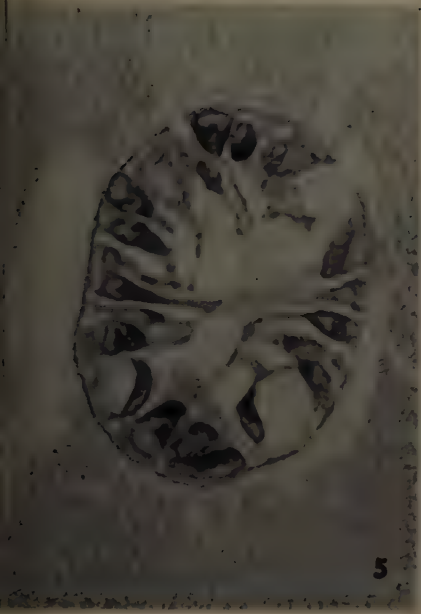
No characteristic cellular appearance or arrangement was noted in the f effusions examined that were associated with neoplasms of the pancreas biliary tract. In those effusions derived from adenocarcinoma of the col the cells were usually arranged as small compact groups and, occasionally, clumps in which the outermost cells had their long axes perpendicular to t outer margin of the clump. Some of the cells showed a signet-ring form d to accumulation of mucicarmine positive material. The cytoplasm of t neoplastic cells was abundant and acidophilic. An occasional fluid show distinctive groupings that closely resembled sections of single glands in aden carcinoma of the colon (FIGURE 5).

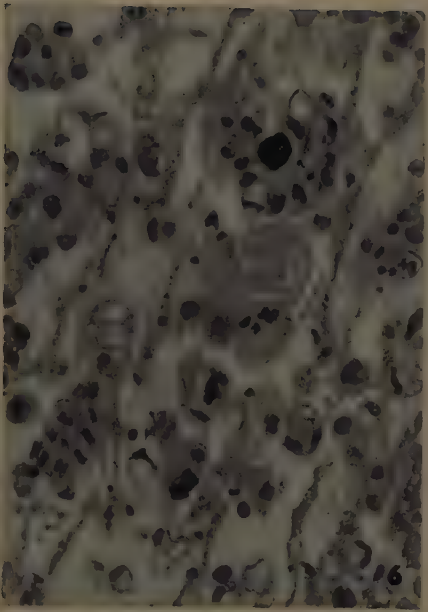
Neoplastic cells were present in over 70 per cent of the effusions associat with carcinoma of the stomach. These cells were usually arranged in so type of aggregate or in aciniform structures. In those fluids derived from u differentiated gastric carcinomas, however, the neoplastic cells were prese as single forms, not as aggregates. These isolated cells were smaller than tho seen with adenocarcinoma of the lung, measuring about 10 to 22 microns diameter. The cytoplasm was scant compared with the isolated cells asso ated with pulmonary cancer. Occasionally, the cytoplasm of the tumor ce was vacuolated. Nuclei were large in relation to the size of the cells, oft multiple, and mitotic figures were frequent.

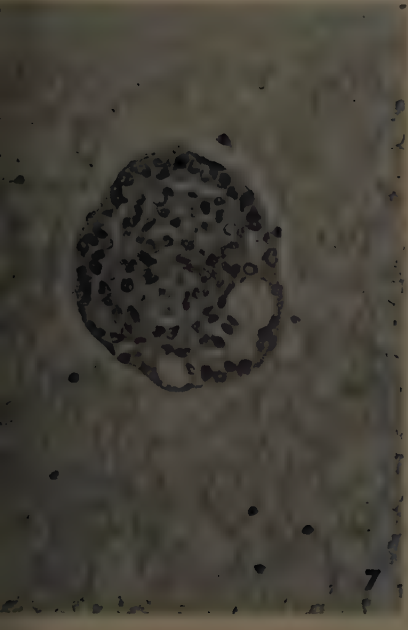
Ovarian Cancer

Cancer cells were present in about 90 per cent of the effusions associat with ovarian carcinomas. The neoplastic cells were numerous, in many spe mens being almost the only cellular element present. The tumor cells we usually clumped or arranged in acini. In effusions derived from papilla serous adenocarcinomas, there was observed a rather characteristic cellul picture. In these fluids, the tumor cells were large and arranged as sing forms, in aggregates, or in acinic structures. The cytoplasm was abunda and often contained vacuoles that displaced and distorted the nuclei, formi large signet-ring cells. The vacuoles contained a mucicarmine positive m terial. The nuclei were irregular, varying in size and shape, and often showi a coarsely granular, densely basophilic chromatin. Frequently, the nuc were multiple. Nucleoli were large and acidophilic. Vacuolization of the exfoliated cells was far more marked than that of the cells in the neoplasm from which they were derived.

Occasional papillary forms were present in fluids, and some of these show a distinct stroma with blood vessels, obviously being fragments of tumor tissu







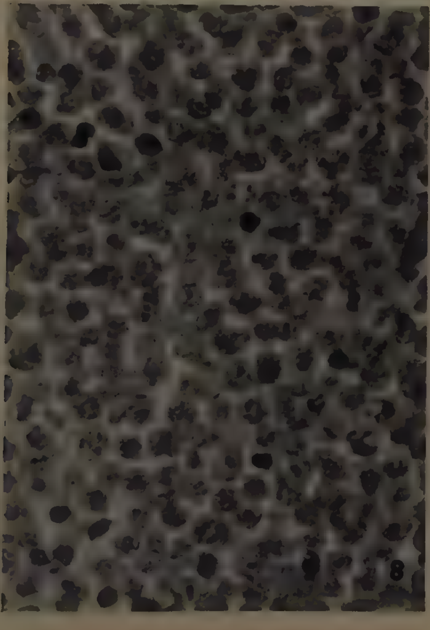


Plate III

FIGURE 5. Adenocarcinoma of colon. Peritoneal fluid. Note alignment cells with their long axes perpen-
ular to outer margin of aggregate. Cytoplasm contains small vacuoles and the entire formation is reminiscent
cellular arrangement in carcinoma of colon. ×600.

FIGURE 6. Ovarian papillary adenocarcinoma. Peritoneal fluid. Note calcific focus in clump of cells at
upper right. Neoplastic cells are irregularly arranged with focal vacuolization of cytoplasm. ×215

FIGURE 7. Adenocarcinoma of breast. Pleural fluid. Large cellular aggregate with palisade arrangement
peripheral cells. ×215.

FIGURE 8. Lymphosarcoma. Pleural fluid. Note uniform densely cellular pattern. Many of the lympho-
es are transitional forms. ×600.

Calcification of malignant tumor cells was not infrequent in ovarian cance The calcified masses were round and laminated, often present within a clum of neoplastic cells. Calcified foci in tumor cells were observed only in effusio associated with ovarian carcinoma (FIGURE 6).

Mammary Adenocarcinoma

In effusions associated with mammary adenocarcinoma, the tumor ce were usually arranged in aggregates that varied from a few cells to large form tions with an irregular pattern. In the latter, the peripheral layer of ce was arranged in a palisadelike fashion, while the central cells showed no pa ticular orientation (FIGURE 7). Acinic forms were present in about one ha of the effusions containing tumor cells and, frequently, they were compose of uniform cells forming a large circular arrangement about a central spac In some cases, these large acini resembled the forms seen in effusions in whi mesothelial cells were arranged as pseudoacini. The cells varied from rath small to moderate in size. The cytoplasm was acidophilic, finely granul and occasionally vacuolated. In only one effusion was a finely vacuolat fatty degeneration of the cytoplasm present in the neoplastic cells.

Malignant Tumors of the Uterus

The types of lesions represented in this group were so variable that no d tinctive appearance was noted.

Malignant Lymphomas

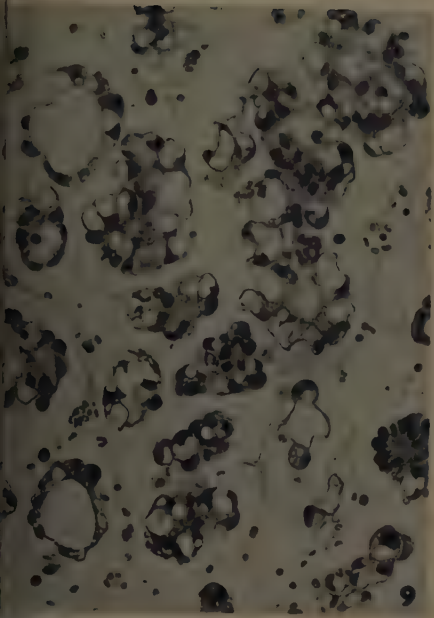
In about 45 per cent of the effusions associated with lymphosarcoma a lymphatic leukemia, an abnormal cellular picture was present. This co sisted of numerous cellular forms, most of which were small lymphocytes some fluids while, in others, there were also medium-sized or large lymphocyt present (FIGURE 8). Mitotic figures were noted in about one third of the e fusions showing this picture. Associated erythrocytes, neutrophils, and mes thelial cells were rare, the predominant cellular form being the lymphocyte varying stages of maturity.

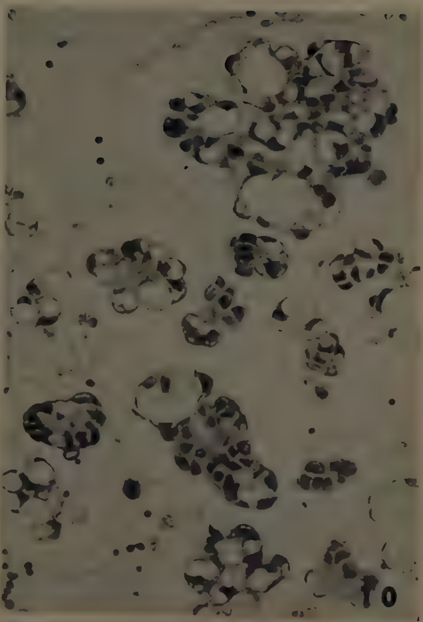
Cells diagnostic of granulocytic leukemia were present in about 65 per ce of the effusions associated with this disease process. Myelocytes were t predominant form, although more immature cells with large round nuclei a scant cytoplasm were sometimes present. In several cases, megakaryocyt were prominent. Neutrophils and stab forms were frequently noted.

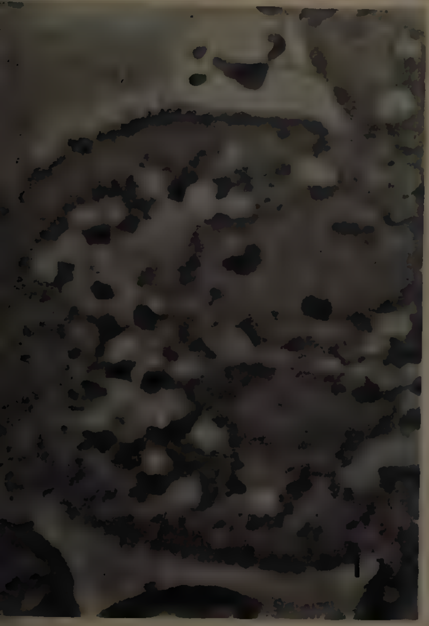
In none of the effusions associated with Hodgkin's disease were definite abnormal cells present. Foot,[5] however, has noted abnormal cells that considers Reed-Sternberg cells. He also observed eosinophilic leukocytes some effusions derived from Hodgkin's disease.

Miscellaneous Tumors

Tumors of which only one or two examples were available have been group together. Only one was of particular interest. A malignant teratoma of t testis with choriocarcinomatous elements showed a distinctive pattern in t associated effusion. The tumor cells were numerous and arranged in aggr







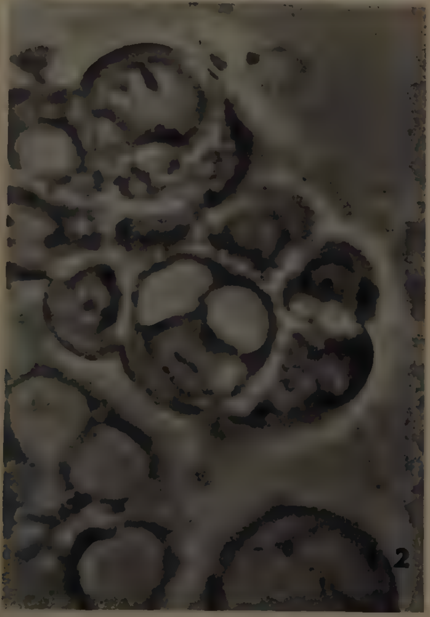


Plate IV

FIGURE 9. Pulmonary adenocarcinoma. Pleural fluid. Numerous cellular aggregates and acinic forms are esent. Cytoplasm shows both fine and coarse vacuoles. Note faint background staining of agar used to hold lls together. Same fluid as FIGURES 11 and 20. ×215.

FIGURE 10. Mammary cancer. Pleural fluid. Numerous neoplastic cells arranged as clumps and acini. ote the appearance of agar in background. Same fluid as FIGURES 12 and 19. ×215.

FIGURE 11. Pulmonary adenocarcinoma. Pleural fluid. Phase microscopic appearance of a single large ll. Note distinct brush border made up of microvilli. Compare with FIGURES 9 and 20. Fixed in osmic acid. 3800.

FIGURE 12. Mammary adenocarcinoma. Pleural fluid. Phase microscopic appearance of typical clump of lls from same specimen as FIGURE 10. Note vacuolization of cytoplasm. ×1100.

gates. Many of the cells were small with a sharp cytoplasmic margin an clear cytoplasm. Interspersed with the small cells were large forms with b zarre and multiple nuclei. Mitotic figures were numerous. Two villusli structures measuring 100 and 150 microns in diameter were present in one the specimens. These villiform structures consisted of a central, loosely a ranged stroma surrounded by flattened cells.

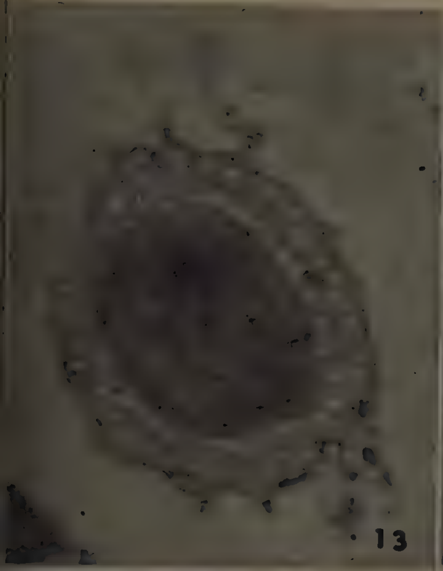
Phase Microscopy of Fluids

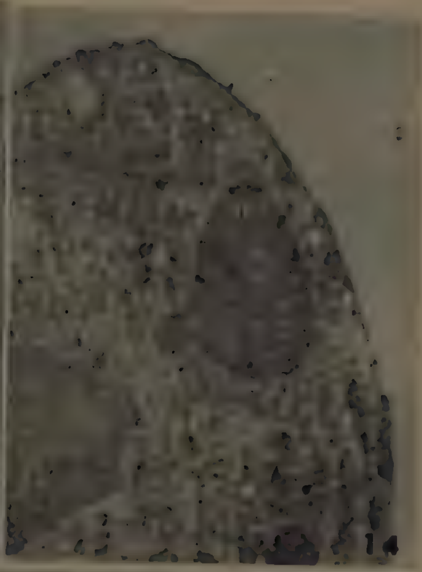
Nine effusions were examined by means of direct smears of cells fixed formalin or osmic acid, as well as by thick sections of methacrylate embedd tissue. The utilization of the phase microscope does not replace examinati of hematoxylin- and eosin-stained sections, but does offer some distinct a vantages. First, by the use of phase microscopy, additional information ma be obtained concerning the structure of these cell groups (FIGURE 11). can be seen in this photograph, there are distinct microvillous projections the surfaces of cells derived from an adenocarcinoma of the lung. These micr villi can not be seen following paraffin embedding and staining, even using th oil immersion lens. A paraffin section of the same fluid specimen is shown FIGURE 9, although at a lower magnification. Second, by utilizing wet smea of the sedimented cells following either formalin or osmic fixation, adequa visualization can be obtained with the phase microscope without stainin Thus, in 30 minutes to an hour, a tentative diagnosis can be made, as to eith the presence or absence of tumor cells in the effusion. Erythrocytes, neutr phils, and lymphocytes are readily distinguished from neoplastic cells not on by their sizes but by their cytologic characteristics. Phase microscopy applie to thick sections (0.5 to 5 microns) of methacrylate-embedded material is useful bridge between light microscopy and electron microscopy, giving add tional orientation with the high magnifications obtained by means of the latte

Fine Structure of Cellular Constituents of Effusions as Shown by Electron Microscopy

Thin sections of methacrylate-embedded cells were photographed at initi magnifications of 1000 to 3000 times, and enlargements were made as desire

Lymphocytes. Electron microscopy revealed relatively few details in th lymphocyte that have not been observed by other techniques. The nucl were round to ovoid, with a dense and rather uniformly distributed chromati The cytoplasm was rather scanty with a finely granular ergastoplasm. Mit chondria were few, rather small, and tended to be concentrated in one portic of the cell. The plasma membrane of the lymphocyte showed irregular sho projections, which were never long or extensive. A rare cell showed a sing osmiophilic inclusion in the cytoplasm, probably representing lipid. In th study, the cells examined were those free in an effusion, so that the poss bility of degenerative or autolytic changes had to be considered. On the bas of the cells so far studied, it is difficult to distinguish with certainty the sma monocyte from the lymphocyte. The first changes noted with degeneratio were coarse clumping of the nuclear chromatin with central pale nuclear fo A typical lymphocyte is shown in FIGURE 13.





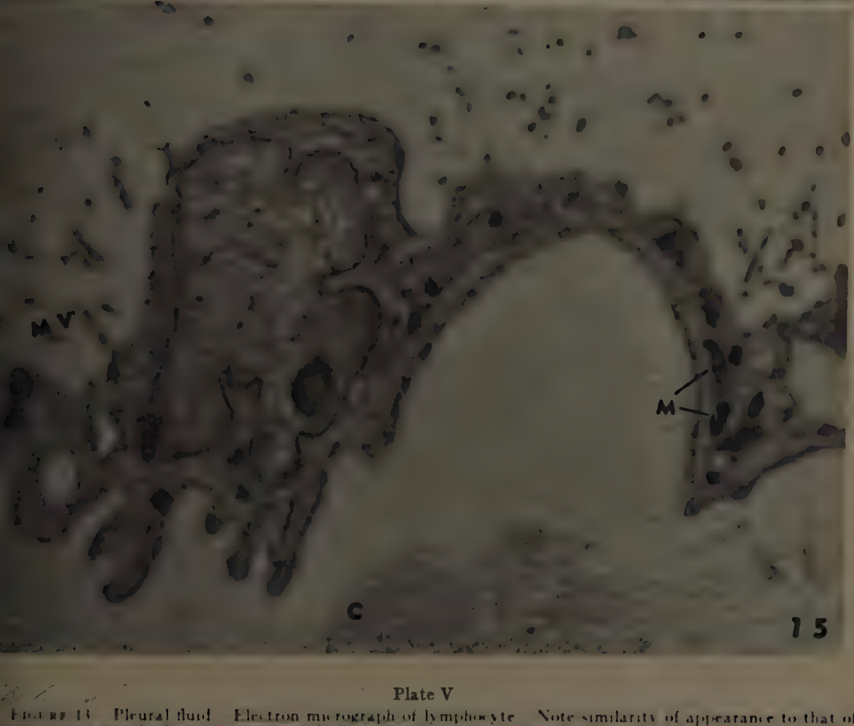


Plate V

Figure 13. Pleural fluid. Electron micrograph of lymphocyte. Note similarity of appearance to that of
mphocyte as seen by light microscopy. Uniform finely granular chromatin in nucleus. Scant cytoplasm with
ely granular and vesicular ergastoplasm. Plasma membrane shows a few fine projections. Mitochondria not
n in this section. ×12,000.

Figure 14. Electron micrograph of portion of neutrophil. Smooth plasma membrane, ovoid shape. Nu-
cus is multilobed, two portions being seen here. Cytoplasm filled with granules, granular ergastoplasm, and
tochondria which are difficult to distinguish from granules at this magnification. Compare with eosinophil,
ure 17. ×12,000.

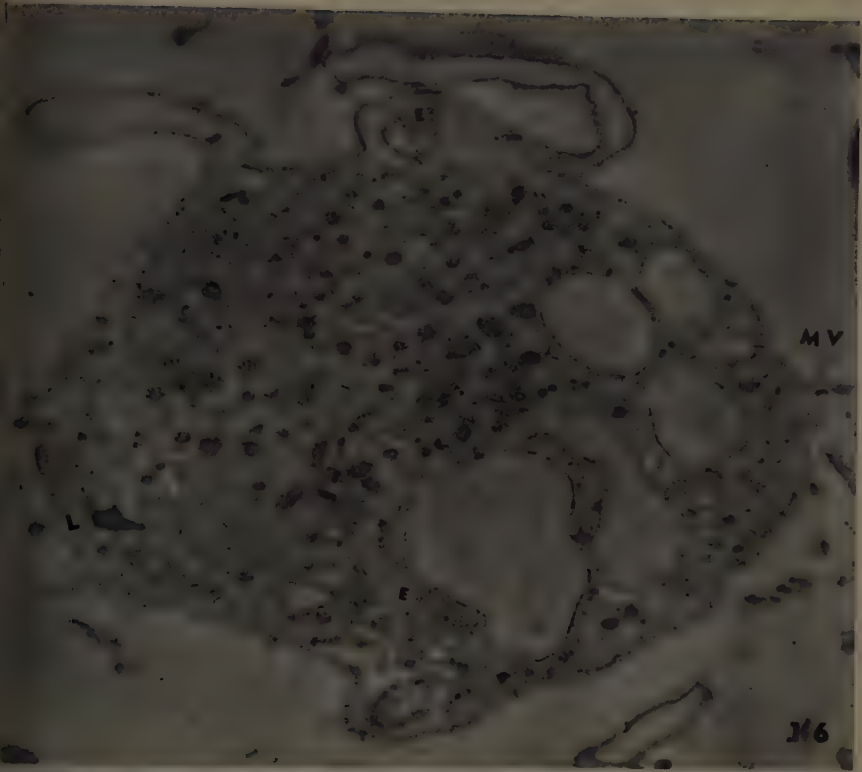
Figure 15. Pleura of mouse. Electron micrograph of normal mesothelial cells. Note the complicated free
face of the cells with microvillar projections of the plasma membrane and cytoplasm (MV). Many of the
rovilli have been cut in cross section and are seen as small round foci free above the cell surface. Mitochondria
are numerous. Note attenuated cytoplasm in portion of cell not occupied by nucleus. Nucleus is irregular
h prominent nucleolus. Basement membrane separating mesothelial cells from underlying collagen (C) is
tinct. ×12,000.

Eosinophils. The eosinophilic leucocytes showed a distinctive appearan
(FIGURE 17). The plasma membrane was rather smooth. The cytoplasm
inclusions were striking, consisting of numerous large, ovoid, and closely plac
granules. Occasional lipid inclusions were present in some cells. The ergast
plasm was finely granular and, in some cells, a faint membranous compone
was also noted. Mitochondria were small and relatively few. The nuc
were bilobed and, frequently, eccentrically placed in the cell. The nucle
chromatin was coarsely clumped in comparison with that of the lymphocy

Neutrophils. Many of the neutrophils present in effusions showed beginni
degeneration. The cells were somewhat smaller than mesothelial cells, a
the nuclei were multilobed so that, in thin sections, they appeared as seve
small individual nuclei. The plasma membrane of the cell was often smoot
although pseudopodia occasionally were present. Vacuolization of the cyt
plasm occurred in some cells, but it was never as prominent as in the mes
thelial cells. The cytoplasmic ergastoplasm or endoplasmic reticulum had
fine vesicular component and an associated finely granular component. T
granules of the neutrophil were less than a tenth as large as those of the eosin
phil, were less osmiophilic, and tended to be uniformly ovoid (FIGURE 1
The mitochondria were small and somewhat elongated.

Mesothelial cells. Normal mesothelial cells from the pleura of a mou
were examined. The mesothelial cells varied considerably, in some foci bei
flattened while, in others, the cells were irregular with thinning of the cyt
plasm only in the portion of the cell not occupied by the nucleus. The cyt
plasm showed a distinct granular and membranous ergastoplasm with rath
large, dense mitochondria. Cytoplasmic vacuoles were present. The mo
striking finding in the mesothelial cell was the presence of numerous micr
villi on the surface of the cells. The microvilli consisted of small protrusio
of the cytoplasm surrounded by the plasma membrane (FIGURE 15). T
basement membrane separating the mesothelial cells from the underlyi
collagen was distinct and, in contrast to that of most epithelial cells, was qui
irregular in outline. The nuclei were irregular in shape with distinct nucle
and a granular chromatin. The nuclei were large in relation to the size of t
cell, although this was more apparent than real, since much of the cytoplas
was attenuated.

Mesothelial cells were numerous in the effusions examined. The mo
common appearance was that of a cell about twice the size of a neutrop
with several elongated rather pale pseudopodia (FIGURE 16). Microvilli we
occasionally present on the surface of the cell. There were numerous vacuo
present within the cytoplasm. In some of the cells, it was apparent that r
blood cells had been phagocytosed. An occasional lipid inclusion was seen
many of the mesothelial cells. Ovoid granules of moderate density were pre
ent. Some of the granules of intermediate size were of lesser density a
suggested the precursors of vacuoles. The ergastoplasm was distinct. Mit
chondria were small and elongated. In some cells, corresponding to the sign
ring forms of light microscopy, there was a single, large, clear vacuole wi
compression of the nucleus to one margin of the cell (FIGURE 18). The nuc
of mesothelial cells were frequently not in the plane of section but, when see
were irregularly ovoid. Nucleoli were sometimes present.



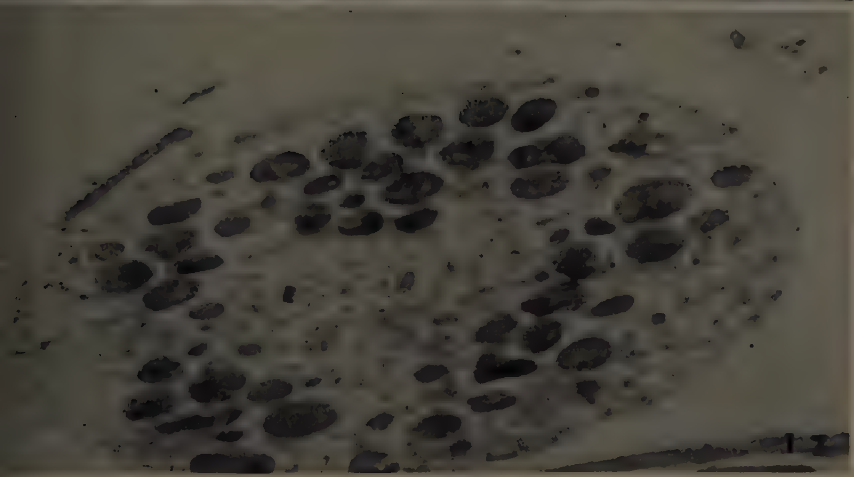


Plate VI

Figure 16. Exfoliated mesothelial cell in pleural effusion as seen in electron micrograph. Note long blunt eudopodia. On right, a few microvilli (MV) are seen. Cytoplasm contains mitochondria and a few granules. ere is a single lipid inclusion (L) present at the lower left. A number of vacuoles as well as slightly smaller oid structures containing a slightly more dense material are present. The ergastoplasm (E) shows a fine osely placed membranous component associated with fine granules. The nucleus is not included in this section. 12,000.

Figure 17. Electron micrograph of eosinophil. This section does not pass through the nucleus. Note rge ovoid osmiophilic granules and compare with smaller granules of neutrophil (figure 14). Note smooth asma membrane of cell. The ergastoplasm is evenly distributed (E). ×12,000.

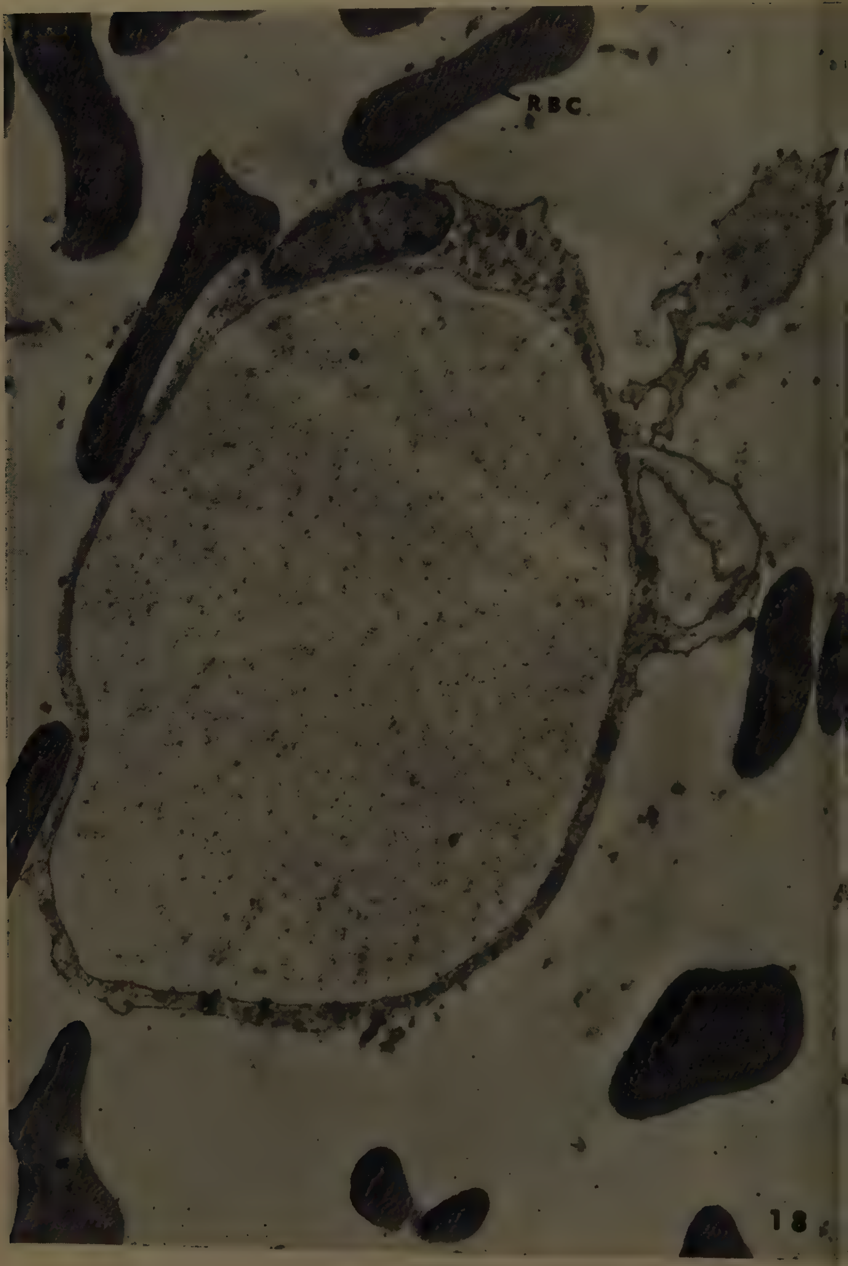
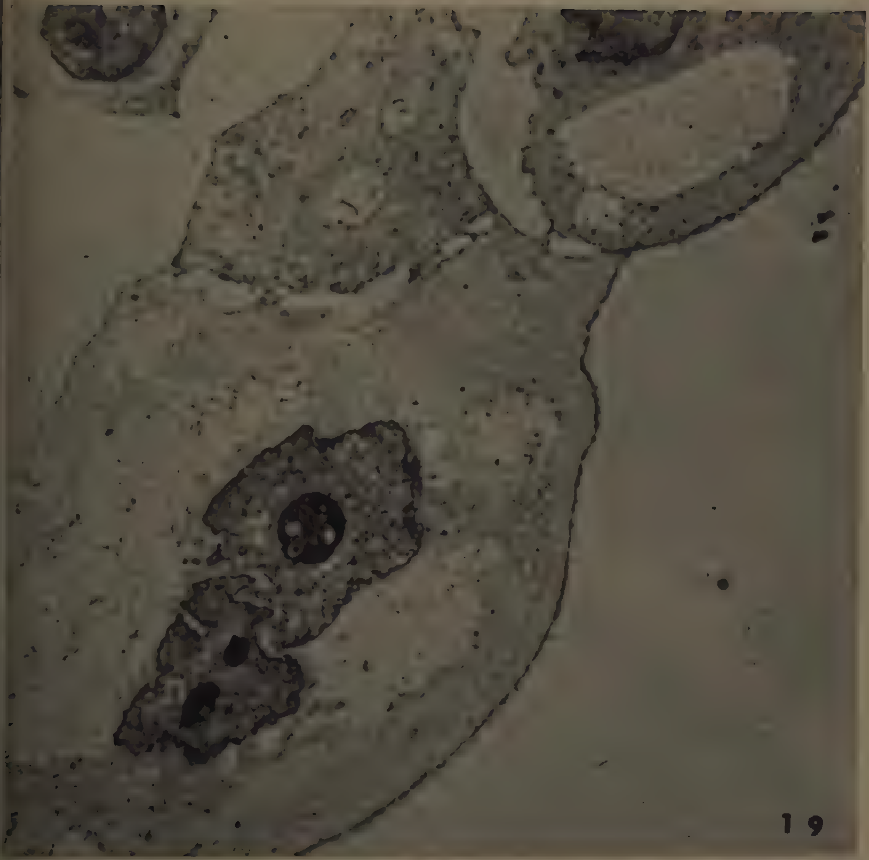


Plate VII

FIGURE 18. Electron micrograph of mesothelial cell of pseudosignet-ring type. Compare with FIGURE Note single, large vacuole compressing nucleus to one margin of cell. Note mitochondria in cytoplasm a distinct pseudopodia. ×7,500.



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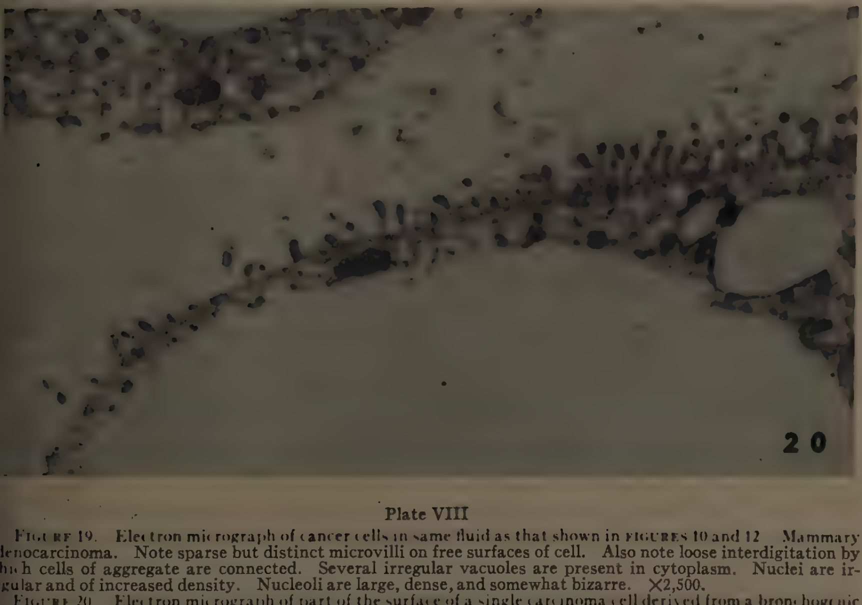

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Plate VIII

FIGURE 19. Electron micrograph of cancer cells in same fluid as that shown in FIGURES 10 and 12. Mammary lenocarcinoma. Note sparse but distinct microvilli on free surfaces of cell. Also note loose interdigitation by hich cells of aggregate are connected. Several irregular vacuoles are present in cytoplasm. Nuclei are irgular and of increased density. Nucleoli are large, dense, and somewhat bizarre. ×2,500.

FIGURE 20. Electron micrograph of part of the surface of a single carcinoma cell derived from a bronchogenic lenocarcinoma. This is the same fluid specimen as that shown in FIGURES 9 and 11. Note complicated plasma embrane with numerous distinct and uniform microvilli. These correspond to brush border demonstrated by hase microscopy in FIGURE 11. Many large and small vacuoles are present in the cytoplasm. ×12,000.

Neoplastic cells. Tumor cells derived from an adenocarcinoma of the lun and from a mammary cancer were examined by electron microscopy. Th cells were large, so that only single cells or parts of clumps of cells were photo graphed. The most striking finding in both types of tumor cells was the com plicated plasma membrane. In the carcinoma metastatic from lung, the ce surface was closely covered by distinct microvilli, as is shown in FIGURE 20 There were numerous large and small vacuoles present in the cytoplasm The mitochondria were not prominent. The nuclei were large and irregula with macronucleoli.

Cells from the adenocarcinoma of the breast showed a similar surface, bu with shorter and more sparse microvilli (FIGURE 19; FIGURES 10 and 12 show cells from the same specimen as demonstrated by light and phase microscopy The vacuoles in the cells of mammary cancer were usually large and singl The nuclei showed bizarre forms. The interdigitating arrangement betwee individual tumor cells was interesting, and was similar to that observed be tween cells in the normal mammary gland.

Summary

Ehrlich's work first stimulated the staining of fixed cells in effusions, whil the embedding and sectioning of cell blocks was reported first by Bahrenburg and, later, by Mandlebaum.[8] Many excellent studies of effusions have bee reported, among which must be mentioned those of Foot,[4, 5] Saphir,[9] Schlesin ger,[10] Wihman,[11] and Zemansky.[12]

In effusions of long duration, the mesothelial elements frequently showe proliferation with unusual groupings of cells to form rosettes, clumps, pseudo signet-ring forms or, more rarely, acini (FIGURES 1 and 2). At times, the acin form arrangements of mesothelial cells were extremely difficult to distinguis from neoplastic cells. In the presence of tuberculosis, numerous lymphocyte were occasionally seen in the effusions. When the lymphocytic cells comprise a majority of the formed elements present in a tuberculous effusion, the latte was distinguished from an effusion secondary to lymphosarcoma only wit difficulty.

The presence of malignant tumor cells in a serous effusion is evidence o metastatic spread of the tumor. Determining the etiology of an effusion however, is a necessary step in the study of the patient, since not all effusion in individuals with known malignant tumors are necessarily secondary to th neoplasms. Similarly, it is often helpful to be able to suggest the possibl site of origin of neoplastic cells in an effusion. This has been discussed pre viously by both Foot[5] and by us.[7] We have found that some of the fluids de rived from adenocarcinoma of the lung, ovarian carcinoma, lymphosarcoma and lymphatic and granulocytic leukemias are readily recognized by thei cellular appearance and arrangement. Less often, effusions derived fro mammary and gastrointestinal lesions can be recognized.

We wish to stress the applicability of phase microscopy to rapid diagnosi of neoplastic elements, as well as to recognition of details not visible in staine sections. By the use of agar as a medium to hold the cells together, mor rapid and easier processing of cells was possible for light and electron micros opy. By the use of electron microscopy, it was possible to begin studying he finer structure of the complicated plasma membranes of neoplastic cells, s well as the details of the cytoplasmic constituents.

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ASPIRATION BIOPSY: TECHNIQUE AND APPLICATION*

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Introduction

The purpose of this paper is to review the technique and application of a piration biopsy as practiced at Memorial Center, New York, N. Y., since th original reports by Martin and Ellis,[49, 50] Ferguson,[22] Coley, Sharp, and Ellis Sharp,[64] and Stewart.[70]

By aspiration biopsy is meant the withdrawal of cells or small bits of tissu through a needle by means of negative pressure. This would include bon marrow aspiration. Needle biopsy of the liver is somewhat different from th usual aspiration biopsy in that a Silverman needle is employed that actual cuts away a small bit of tissue without the use of negative pressure. Becau of ample reports on bone-marrow aspiration and on liver needle biopsy, the procedures will not be discussed in this paper.

With the popularization of cytological diagnosis during the past few year there has been increasing interest in aspiration biopsy, reflected by the nu merous articles on the subject. The references listed in a recent report b Smetana[66] on this subject will serve to augment those given here.

In 1867, Lücke and Klebs[43] described malignant cells in smears of peritone fluid obtained by trocar in a case of carcinoma of the pancreas with metastas to the omentum.

Bahrenburg[2] in 1896 reported his results after examining ascitic and pleur fluid from three patients. Grieg and Gray,[29] in 1904, performed aspiratio of nodes in order to isolate the etiological agent of trypanosomiasis. Whi and Pröscher[75] in 1907 recorded their unconfirmed observations of spirochet in juice aspirated from lymph nodes in various diseases. In 1909, Horder reported upon lung puncture for diagnosis. Ward[74] discussed node punctu in 1914 and suggested that the appearance of cells obtained by puncture nodes might aid in the diagnosis of lymphoblastoma. In 1914, Chatard an Guthrie[6] reported upon a case of trypanosomiasis diagnosed by node aspira tion. Guthrie,[30] in 1921, made a serious attempt to study cells obtained b aspiration from lymph nodes.

In 1922, Ellis[21] recorded three cases of carcinoma diagnosed by examinatio of pleural fluid. In 1927, Dudgeon and Patrick[19] recorded a study of 2 tumors examined by direct smears and, during the same year, Forkner[25,] reported upon a study of node aspirates.

Following this, around 1926, Doctor H. Martin,[47] with the cooperation Doctor F. W. Stewart and of E. B. Ellis of Memorial Hospital, began wha has now become a rather routine procedure at this institution.

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* The work described in this paper was supported by the Atomic Energy Commission, Washington, D. The photographs were prepared by Robert F. Smith, of the Brookhaven National Laboratory. Most of t photomicrographs are of either 140 or 310 magnification.

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It is a procedure which has been found to be time-saving, efficient, relatively painless, safe, and inexpensive. It requires cooperation between the surgeon and the pathologist, and a knowledge on the clinician's part of what may be expected of the procedure. The interpretation of aspirates, as with other pathological material, is certainly not without pitfalls. It requires experience. It is necessary that a sufficient number of cases be available for both clinician and pathologist to maintain their efficiency. The pathologist must know the clinical setting, the normal cells of the region, and the nature of lesions to be anticipated in the area. Experience may be obtained by making smears from surgical and autopsy material or by doing aspirations without rendering reports until proficiency and confidence are obtained.

At present, about 2,500 aspirations are performed annually on the combined services at Memorial Hospital. Since the late 1920's, when the procedure was initiated, few complications have occurred through its use. There is no doubt that spread along the track may occur but, in most instances, if the lesion proves to be malignant, the track is excised when a breast, for instance, is removed, so that this danger is obviated. In the innumerable instances where the track remains, however, spread along it is so infrequent that documented material of such cases is nearly nonexistent. I have examined the material on one case reported by Crile and Hazard.[15] This was a papillary carcinoma of the thyroid in which a small tumor nodule developed in the needle track. Dolley and Jones[18] have recorded extension along a thoracentesis track. Air embolism may occur as a complication of aspiration of the thorax.

Many cells are obtained in the average aspiration and, therefore, the diagnosis of aspirated material is not based upon three or four cells, but generally upon a large number. In the smears, the cells show loss of cohesion, large and hyperchromatic nuclei, cytoplasmic vacuolization, large nucleoli, and abnormal nuclear-cytoplasmic and nuclei-nucleoli ratios. Occasionally, more specific features, such as mucus secretion, may be evident. The clots are helpful in many instances where the smear is not diagnostic and in making a more definitive diagnosis as to the type of tumor.

Indications and Contraindications

The indications for the procedure have not changed since the original reports. They include: (1) any palpable tumor deep to the surface and covered by normal tissue; (2) a tumor where surgical biopsy is difficult or requires a major procedure; and (3) any deep-seated lesion.

The contraindications for aspiration are lesions that may be in proximity to such vital structures that damage might be serious, as about the large vessels of the mediastinum. Although large vessels and viscera have been punctured, serious consequences have not resulted. For a more extensive discussion of the advantages and limitations of aspiration biopsy, one is referred to an article on this subject by Martin and Stewart.[51]

Aspiration is employed principally in lesions of the breast, head, and neck regions, and of the bone, since lesions in these positions offer the most practical application of this procedure.

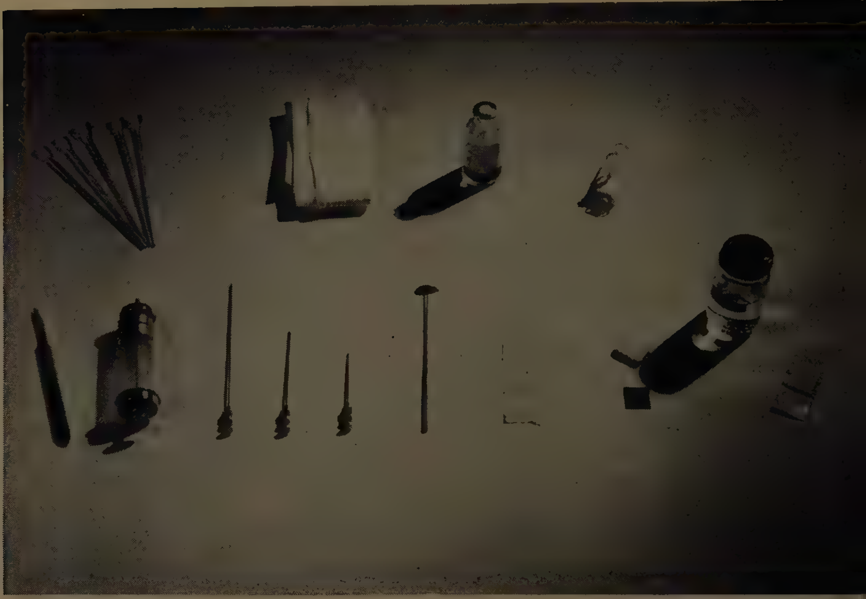

FIGURE 1. Paraphernalia employed in aspiration biopsy.

Technique

Many different needles and apparatus[5, 7, 8, 32, 65] have been employed fo aspiration and needle biopsy. The more simple ones, however, are still preferred. These consist of a record syringe (20 cc.) and 18-gauge needles of different lengths with obturators and rake (FIGURE 1).

The skin over the aspiration site is sterilized and incised with the end of sharp No. 11 Bard Parker blade. The needle is inserted into the tumor whil the tumor is steadied with the opposite hand (FIGURE 2). The obturator i removed and the syringe attached. The plunger is pulled as far out as possible and the needle gently moved in different directions within the tumor in orde to dislodge material (FIGURE 3). After the material is obtained in the syringe the negative pressure is released to obviate splattering of the aspirate in th syringe. With the rake, the material is placed on several slides and *gentl* smeared by approximating two slides and pulling them apart. The remainin material is placed on a small piece of blotting paper or fibrin foam and put i formalin for later paraffin section (FIGURE 4). This is designated as the clot

The smears are allowed to air-dry. This has been found to give satisfactory results. A variety of fixatives has been tried, as well as alcohol-ether fixation while the smears are still wet, but this has not materially helped in diagnosis

Fluid sediments may require albumin-coated slides. The smears are staine with hematoxylin and eosin. Clots are processed as any other biopsy.

The application of the procedure will be discussed in a regional fashion, i view of the arrangement of services at Memorial Center.

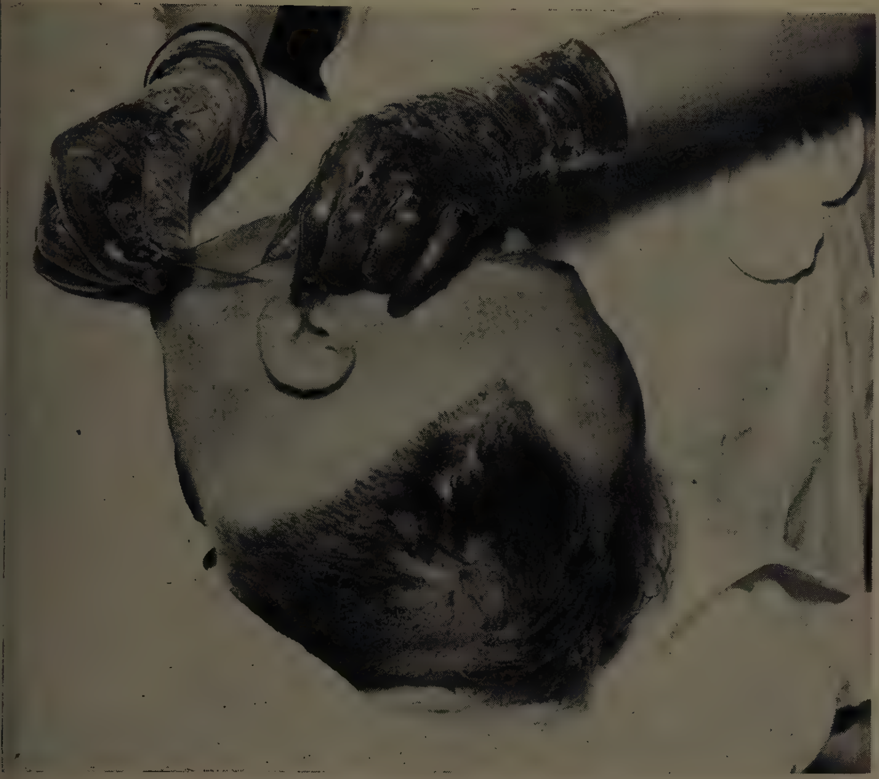

FIGURE 2. Aspirating needle being inserted into tumor.

Breast

Cancer of the breast is one of the most suitable of neoplasms for aspiration. It is readily accessible and there are no major structures that might be damaged accidentally.

It has been the policy of the Breast Service to aspirate breast lesions in the outpatient clinic and on those patients admitted for surgical procedures. In hospitalized patients, aspiration is performed the night prior to the day of operation. The material is submitted to the laboratory and is reported upon the following morning. After a report of carcinoma, a radical mastectomy is performed, thereby obviating a local excision, lengthened anesthesia time, and redraping.

The cells from carcinoma of the breast generally show prominent variation in nuclear size with abnormal nuclear-cytoplasmic ratio, hyperchromasia, and large nucleoli. The cells are usually arranged in small clumps or singly, being less cohesive than normal, or noncancerous epithelium, such as the cells from a papilloma. Occasionally, an aspirate will be composed of smaller neoplastic cells somewhat resembling plasmocytes.

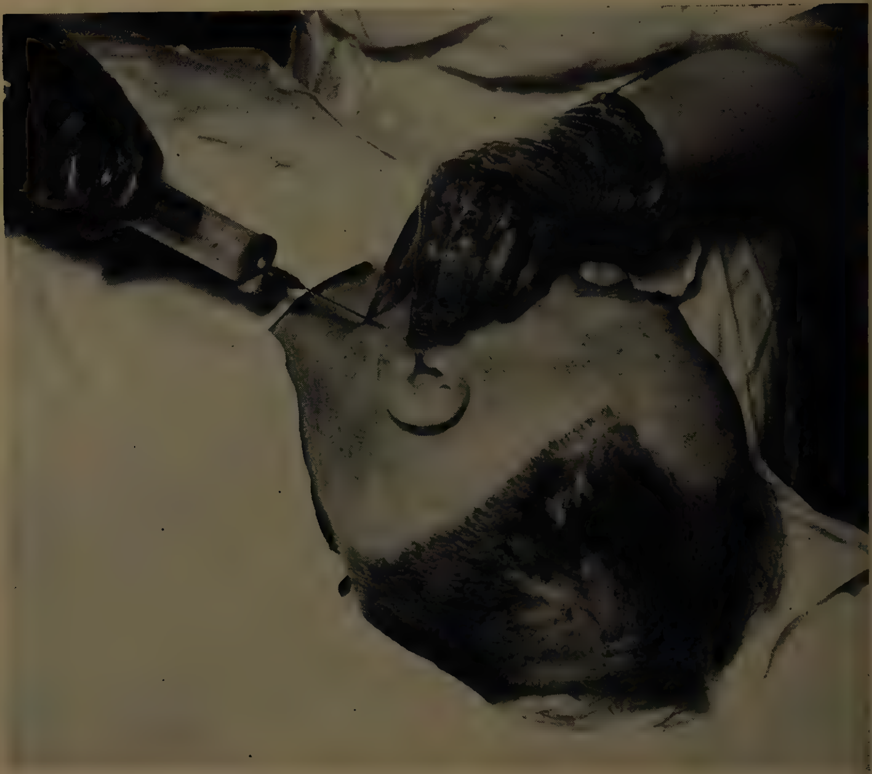

FIGURE 3. Tumor being aspirated.

Smears are much more satisfactory for diagnosis than are the clots in breast aspirations. An occasional aspiration presents difficulties, and a report reading "atypical cells, require frozen section" is rendered. It is, of course, more sound to err on the side of safety if there is any doubt as to the diagnosis of cancer.

It would appear reasonable that an aspiration biopsy of a breast carcinoma would be less likely to disseminate cancer cells to the extent that an incisional or excisional biopsy would. The remote possibility of extension of the tumor along the needle track is of no practical concern, since the breast is removed following diagnosis or, if the lesion is not amenable to surgery, little harm would have been done.

The greater the experience with the procedure, the more useful it becomes. The essential point in aspiration biopsy is to differentiate benign from malignant lesions. Diagnosis by smears of the average carcinoma of the breast is generally an easy procedure after a little experience (FIGURES 5, 6, and 7). There are instances in which the atypical cells are few or only slightly atypical. A situation such as this requires conservatism, since a few atypical epithelial cells may be seen in cystosarcoma phyllodes, papilloma, and sclerosing adenosis.

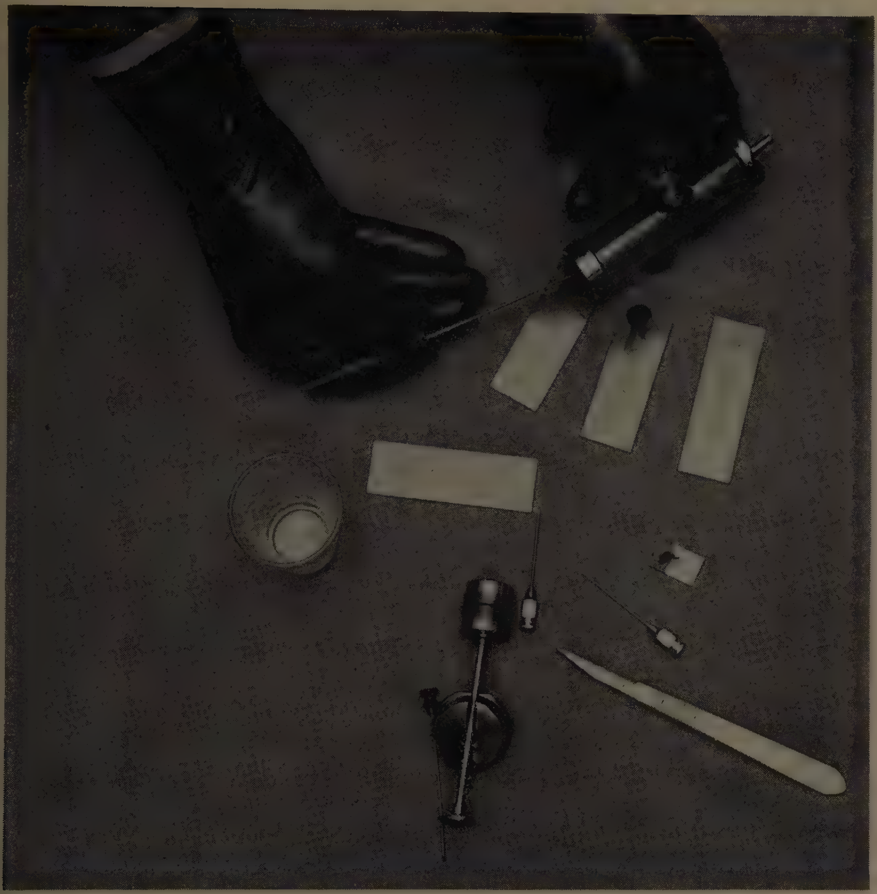

FIGURE 4. Smears and clot being prepared from aspirated material.

When the diagnosis of carcinoma is made, there should not be any doubt that the diagnosis is correct. To compromise will lead to mastectomy for benign lesions. There is a level of cytological atypism, below which one may not safely diagnose carcinoma even though the percentage of error may be small. Any radical mastectomy for a benign lesion is too many for any series. The solution to this is experience with many smears. Papilloma may be suggested and diagnosed by smears when one obtains sheets of epithelial cells with uniform nuclei. Sheets of epithelial cells from fibroadenoma, cystosarcoma phyllodes, and cystic disease (FIGURE 8) may simulate papilloma.

Papillary carcinoma may suggest papilloma superficially. The variation of nuclei in the former, however, will usually enable one to separate the two. Fibroadenoma may be diagnosed if fibrous fronds are found. The epithelial cells may be in sheets but are uniform.

Cystosarcoma may be diagnosed occasionally by the presence of fronds (FIG-

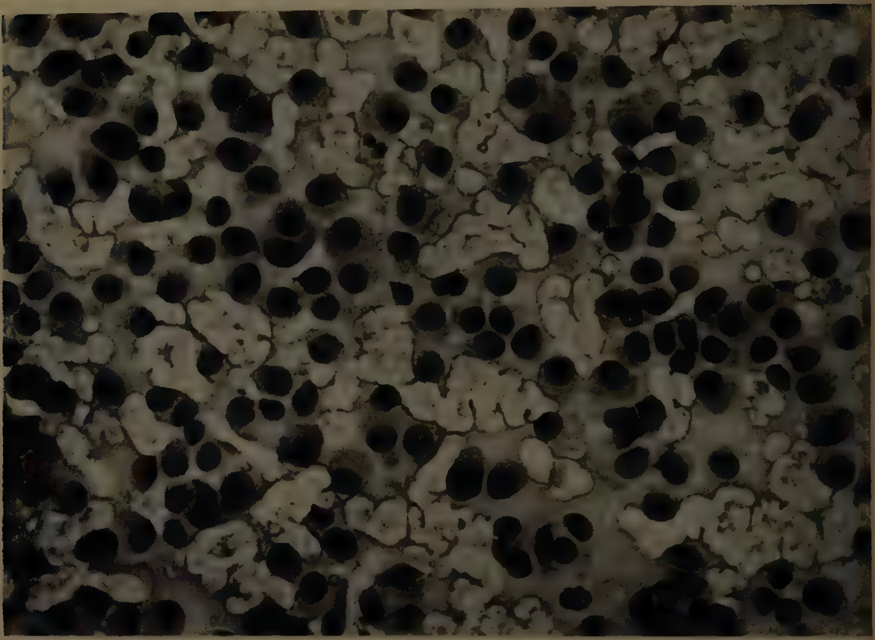

FIGURE 5. Cells obtained from a breast carcinoma. Note lact of cohesion. Smear. ×310.

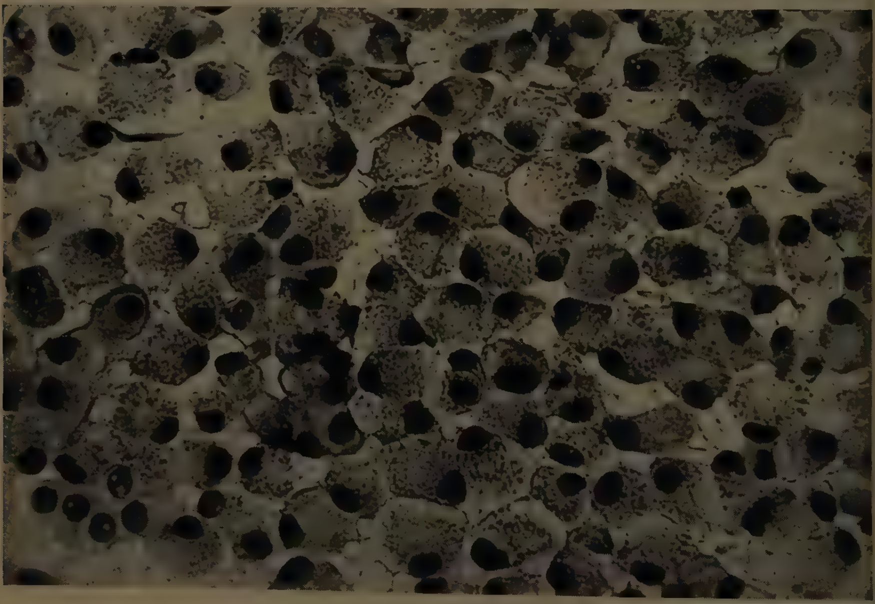

FIGURE 6. Cells obtained from breast carcinoma, mucinous type. Smear. ×310.

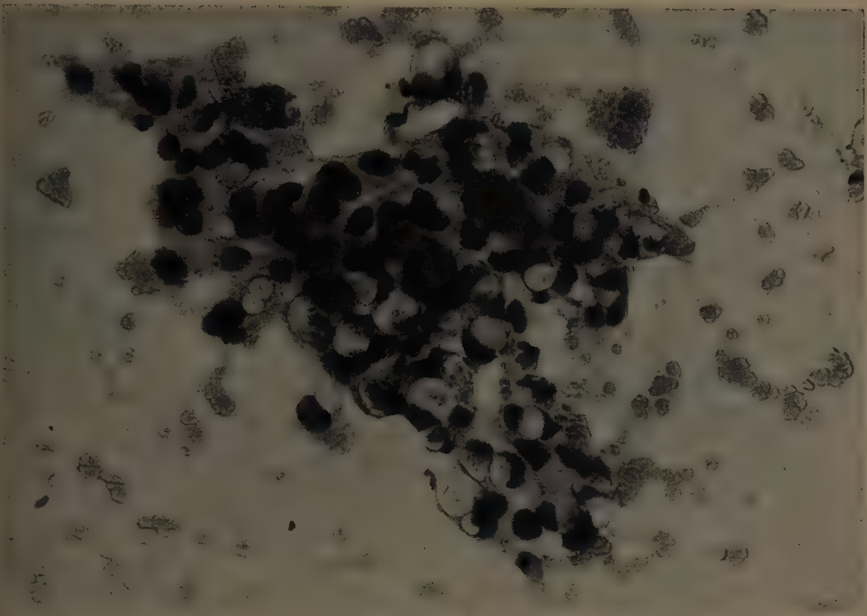

FIGURE 7. Cells obtained from a breast carcinoma. Note mucus in some cells. Smear. ×310.

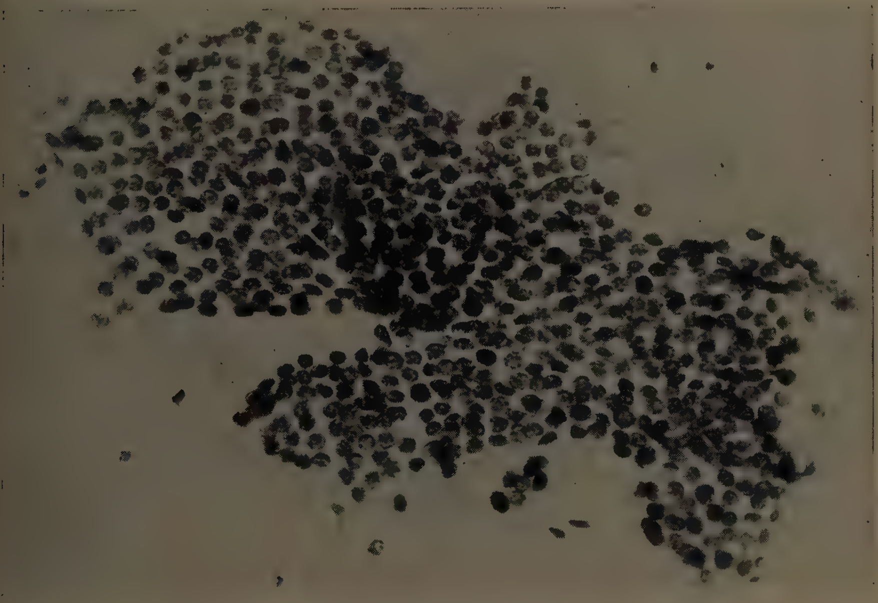

FIGURE 8. Sheet of cells from cystic mastopathy. Smear. ×310.

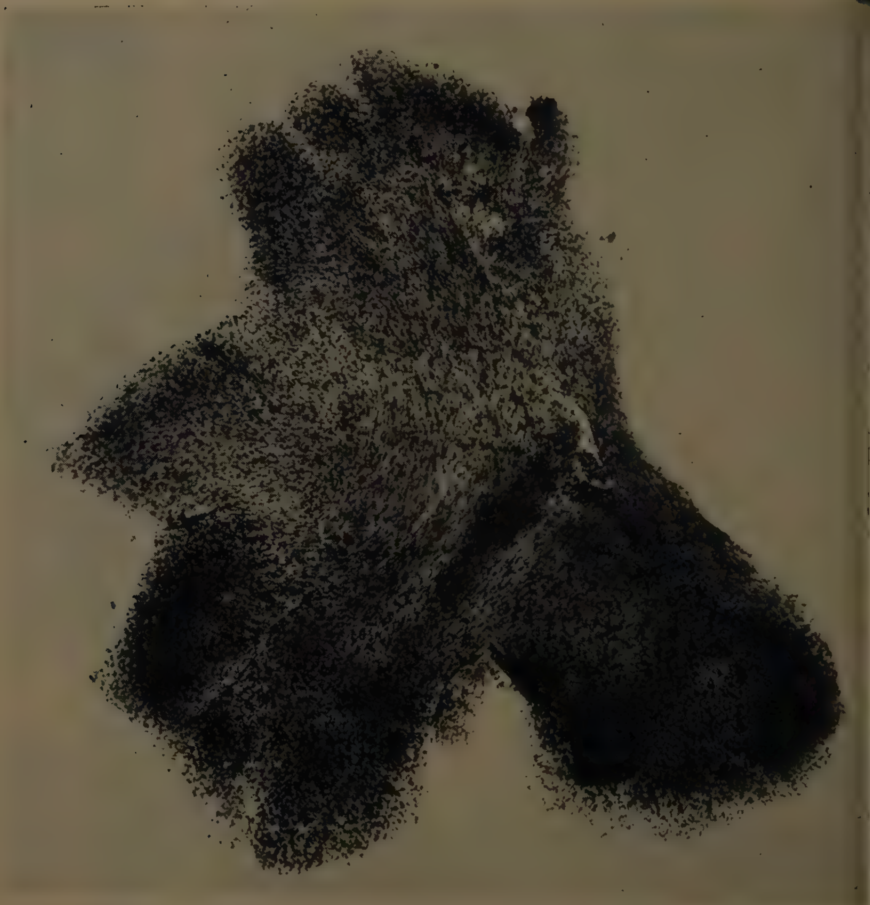

FIGURE 9. Frond aspirated from a cystosarcoma phyllodes. Smear. Low power.

URE 9) and atypical epithelial cells. Rarely, a malignant cystosarcoma may be diagnosed. Other lesions, such as malignant lymphoma, angiosarcoma, and liposarcoma may be diagnosed as a malignant tumor and, occasionally, the histogenesis of the lesion may be suggested.

Aspirates of cysts rarely contain malignant cells, usually only a few benign epithelial and red blood cells. Johnston[35] has recently reported upon the use of aspiration in cysts of the breasts as a therapeutic procedure. It has been similarly employed in the Breast Clinic for many years with good results. Occasionally, cysts may contain large apocrine cells that may cause some diagnostic difficulty, but the cells are usually quite uniform and have a normal nuclear-cytoplasmic ratio. Aspirates from areas of fat necrosis contain macrophages, foreign body giant cells, and leucocytes. A few epithelial cells, usually with uniform nuclei, are encountered. One should be cautious in diagnosing carcinoma in the presence of inflammatory material, since atypical but benign cells may be encountered.

Gynecomastia may be suggested by the presence of sheets of benign epithelial cells. Mastitic aspirates will show many inflammatory cells.

Of 1,579 breast carcinomas seen in one five-year period, 806 aspirations were performed by experienced residents, and a correct diagnosis was obtained in 90.1 per cent. In the remainder (9.9 per cent), inadequate material was obtained for diagnosis. Of the 773 cases of breast carcinoma in which aspiration was performed by less experienced residents, there were 156 (20 per cent) failures.[1] The incidence of false positive reports has been nearly nil.

Robbins *et al*,[62] in a recent study made to ascertain whether or not aspiration biopsy of breast cancer is dangerous to the patient, concluded that it seemed to have no effect on long-term survival rates. Kaae[36, 37] has employed trephine biopsy of breast lesions extensively.

Head and Neck

Lesions about the head and neck region, such as those in lymph nodes, salivary glands, antrum, and thyroid, readily lend themselves to aspiration. Enlarged lymph nodes in the presence of a carcinoma may be aspirated in order to plan the surgical procedure properly. A positive aspiration obviates surgical intervention in the neck, which might interfere later with a proper neck dissection. Aspirated material from nodes in metastatic carcinoma occasionally enables one to guess the primary site, such as thyroid, ovary, gut, and so on. Of numerous lesions occurring about the head and neck, many have been aspirated and diagnosed, such as carotid body tumors,[46] neurilemoma,[20] bronchogenic cysts, all types of salivary gland lesions[48] (FIGURES 10, 11, and 12), malignant lymphoma, actinomycosis, various thyroid le-

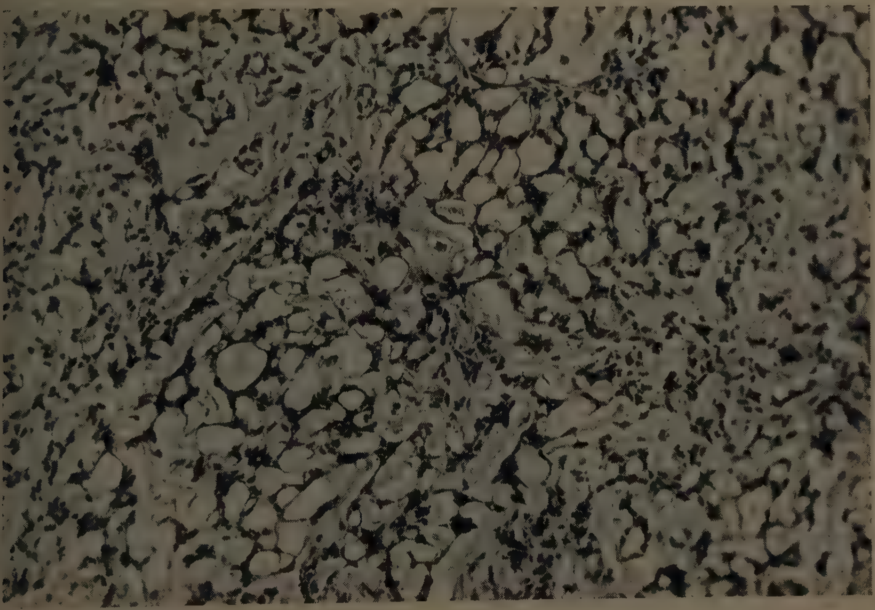

FIGURE 10. Aspirate of an adenoid cystic carcinoma from parotid. Clot. ×140.

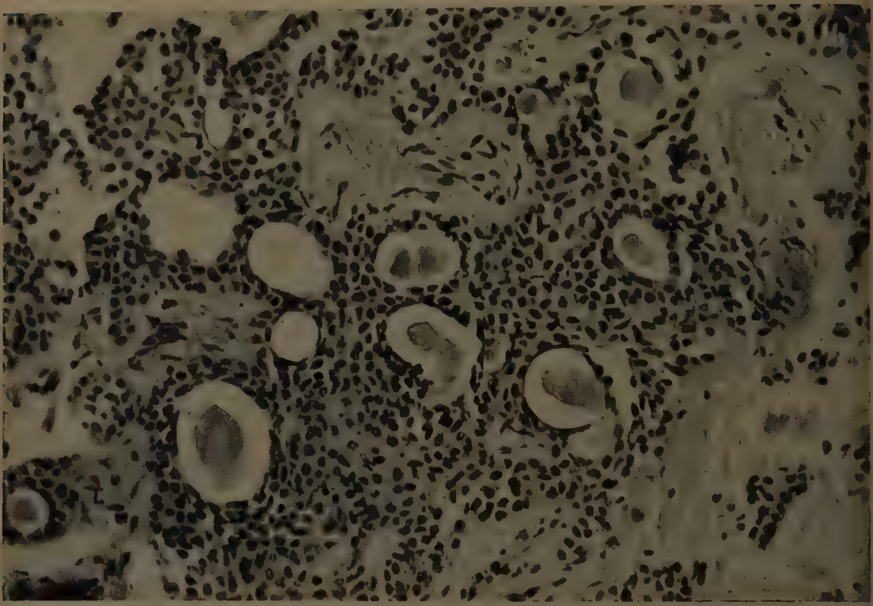

FIGURE 11. Aspirate of a mixed tumor from parotid. Clot. ×140

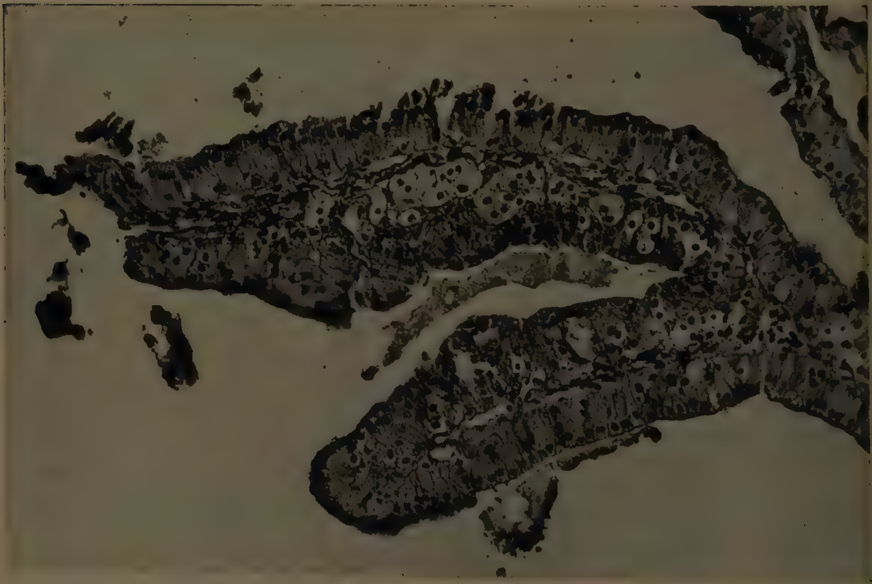

FIGURE 12. Aspirate from parotid showing a papillation of a papillary cystadenoma lymphomatosum. Clot. ×140.

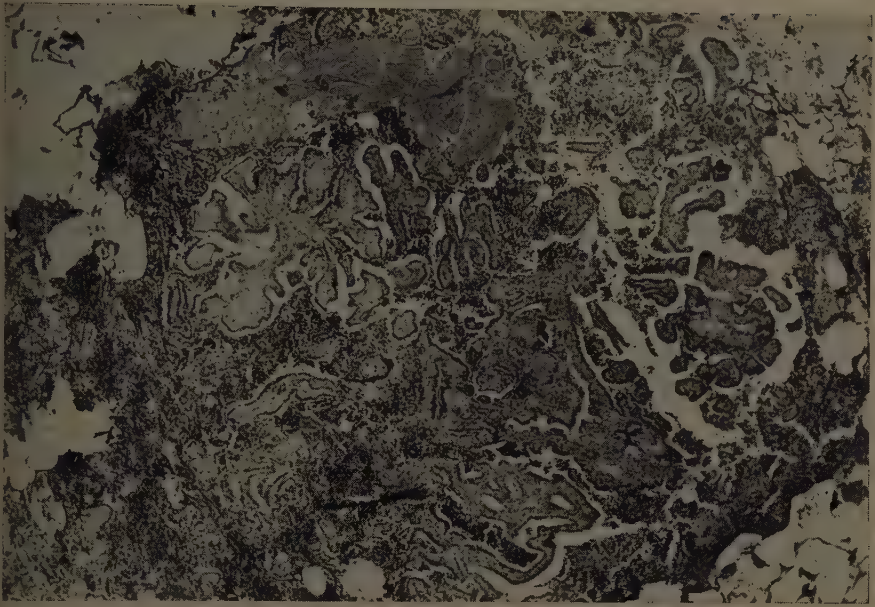

FIGURE 13. Aspirate of a papillary carcinoma of thyroid. Clot. ×27.

sions[68, 16, 41] (FIGURES 13 and 14), antral lesions (FIGURE 15), metastatic carcinomas (FIGURES 16, 17, and 18), and others. Here, again, it is necessary for one to know the site of the aspiration and what lesions may occur in that particular location.

Bone

The Bone Service routinely performs aspiration biopsies and reports about 80 per cent successful results.[4, 67, 13, 31] Valls *et al.*[73] have written a monograph on this subject. A review of 150 cases of multiple myeloma[27] at this institution revealed that 56 were diagnosed by aspiration biopsy (FIGURE 19), excluding sternal aspiration. All types of bone lesions, except the more sclerosing types of osteogenic sarcoma, lend themselves to aspiration. Occasionally, differentiation between a metastatic and a primary lesion may be difficult. Also, differentiation among lesions such as Ewing's sarcoma (FIGURE 20), reticulum cell sarcoma, and metastatic neuroblastoma may not be possible nor, for that matter, may they be with sections. As always, an adequate history and a knowledge of the clinical features are of great help. Difficulty may certainly arise, as in the giant cell and cartilaginous tumors since, in large paraffin sections, the diagnosis may be difficult. FIGURES 21 to 24 show several aspirates of various bone lesions.

Soft Parts

Many lesions of the soft parts are treated on the Mixed Tumor Service. These include liposarcoma, malignant neurilemoma, rhabdomyosarcoma (FIG-

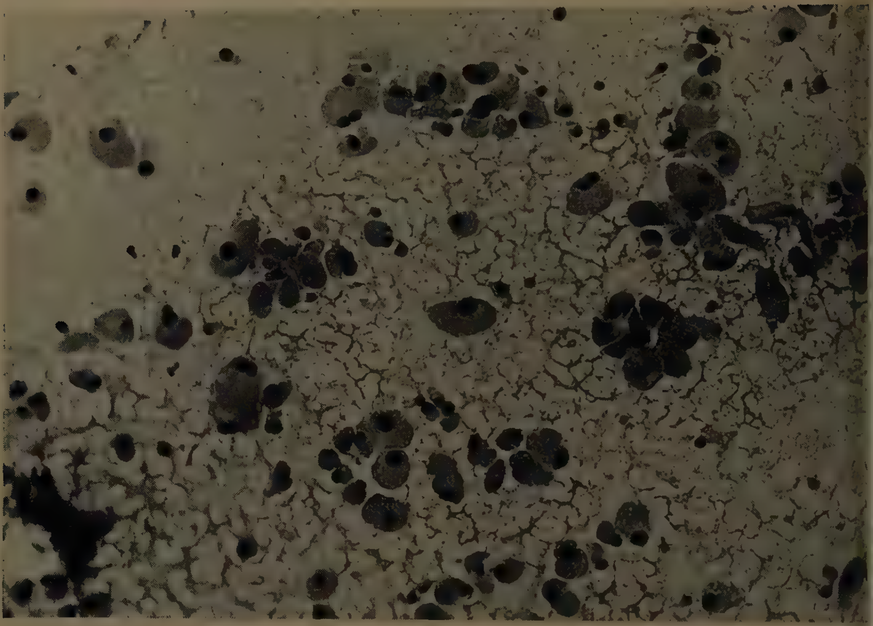

FIGURE 14. Aspirate from a Hürthle cell tumor of thyroid. Smear. ×140.

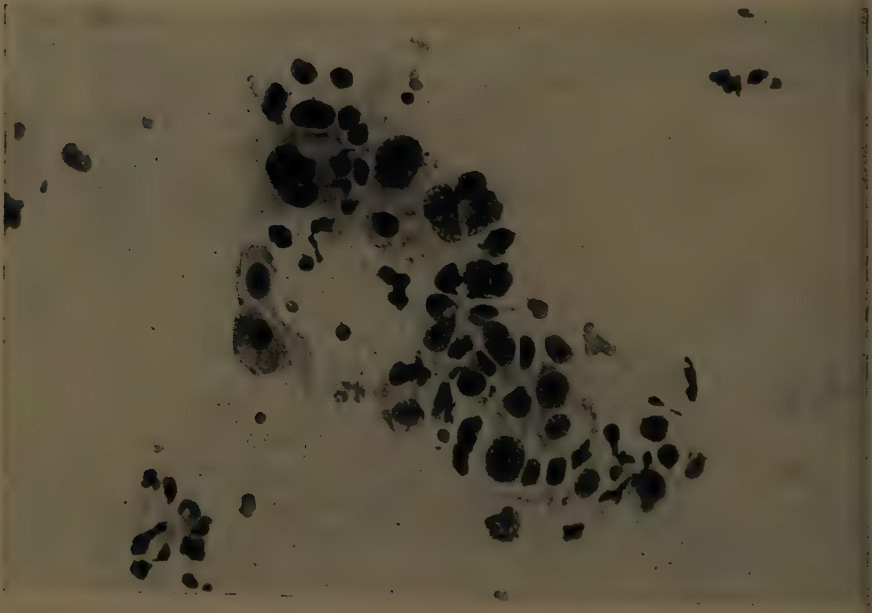

FIGURE 15. Aspirate from a carcinoma of the maxillary antrum. Smear. ×310.

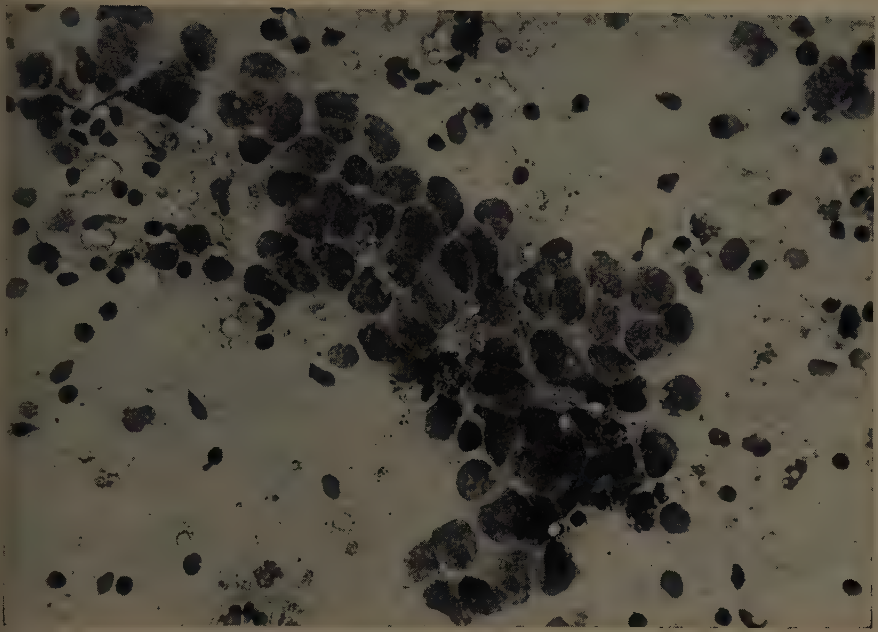

FIGURE 16. Aspirate from a cervical lymph node showing metastatic carcinoma. Known primary of testicle. Smear. ×310.

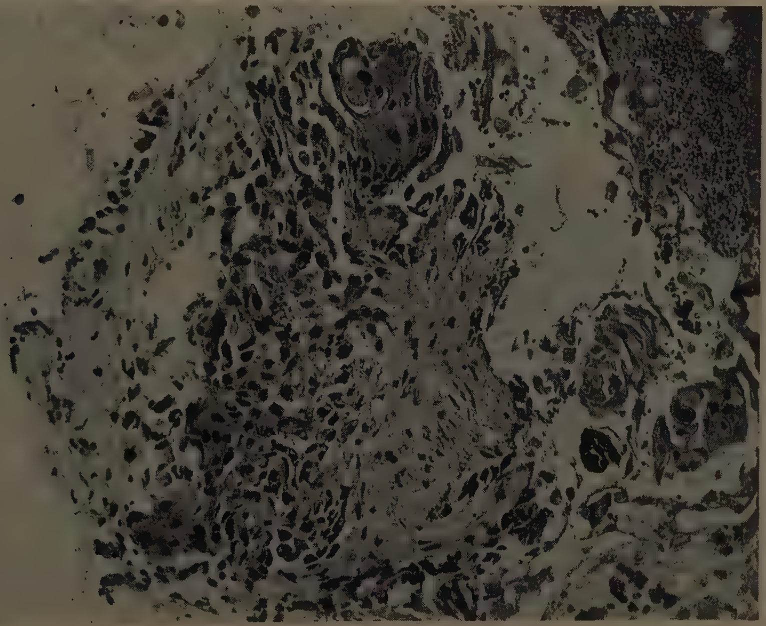

FIGURE 17. Aspirate from a cervical lymph node showing metastatic squamous carcinoma. Clot. ×140.

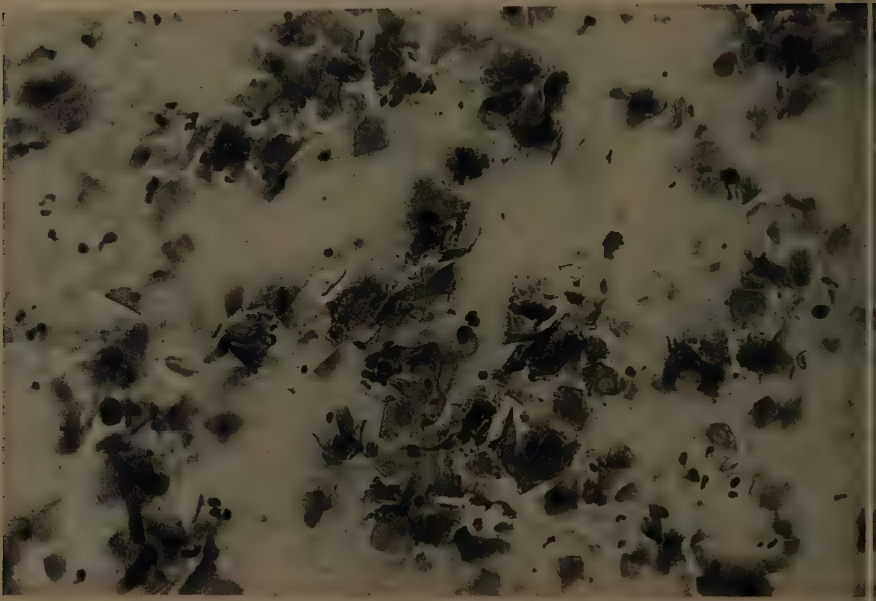

FIGURE 18. Aspirate from a cervical node showing keratinized squamous cells from a metastatic carcinoma. Branchiogenic cyst contents may be similar, therefore malignant cells must be found before classifying this type smear as malignant, unless the clinical setting is consistent. Smear. ×140.

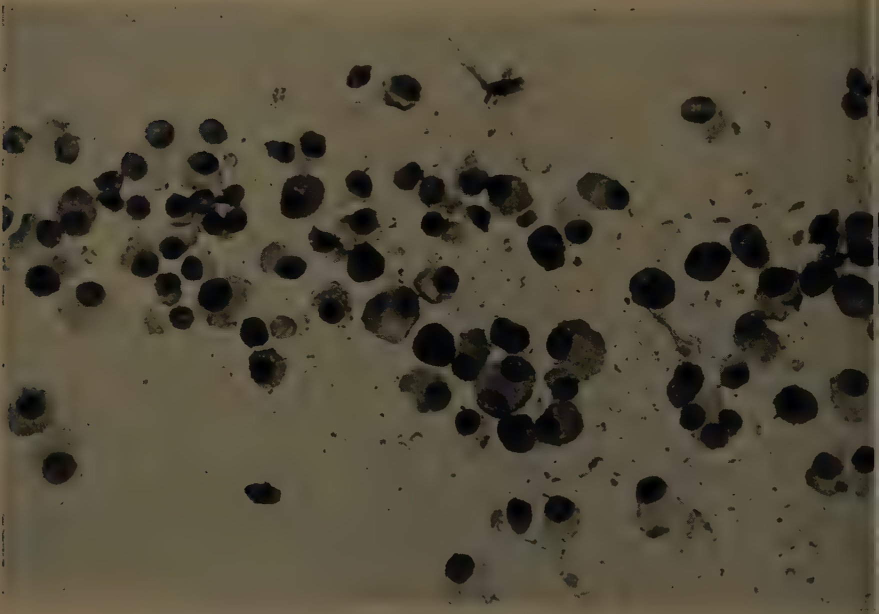

FIGURE 19. Aspirate from bone showing plasma cell myeloma. Smear. ×310.

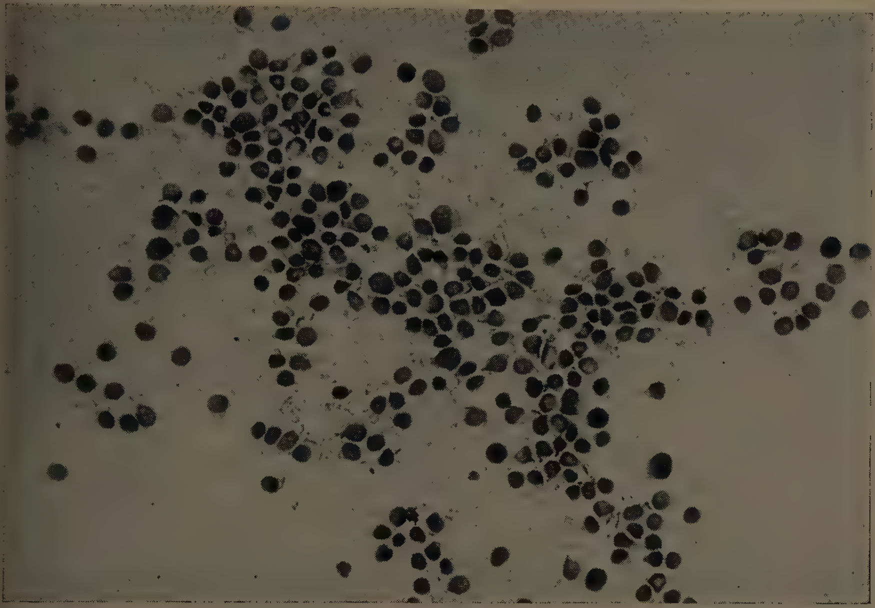

FIGURE 20. Aspirate from a Ewing tumor. Smear. ×145.

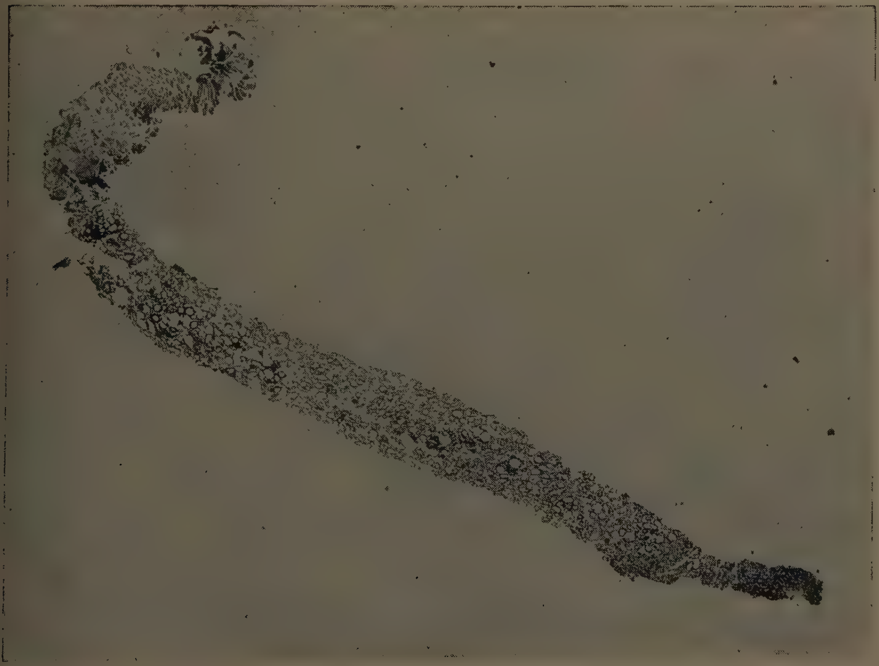

FIGURE 21. Aspirate from sternum showing metastatic follicular carcinoma of thyroid. Clot. ×8.5.

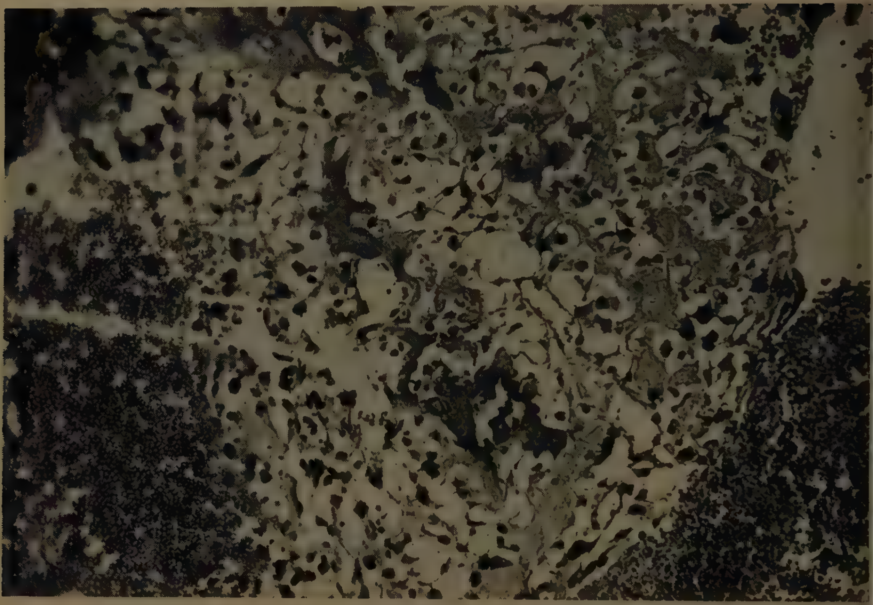

FIGURE 22. Aspirate from humerus showing an osteogenic sarcoma. Clot. ×140.

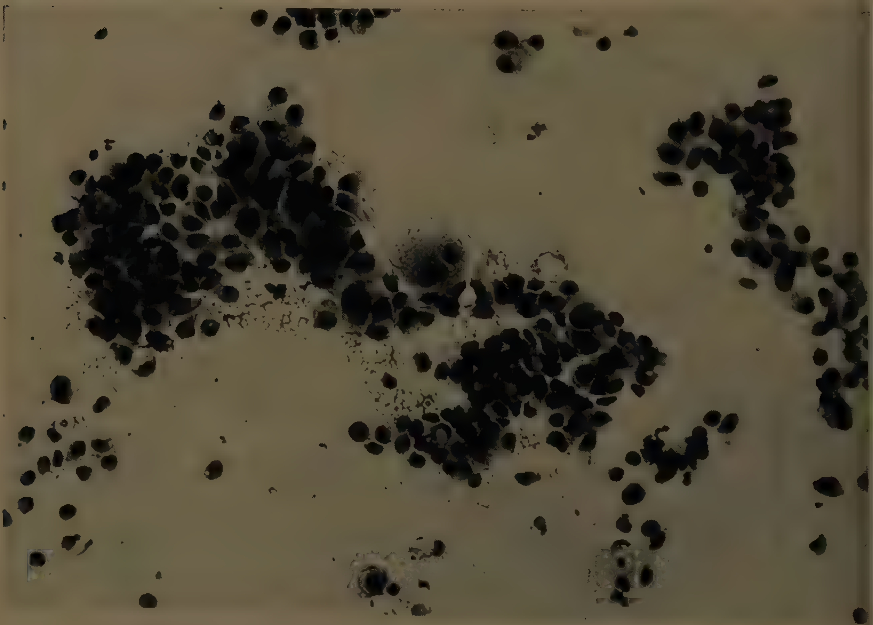

FIGURE 23. Aspirate from bone showing metastatic carcinoma from breast. Smear. ×310.

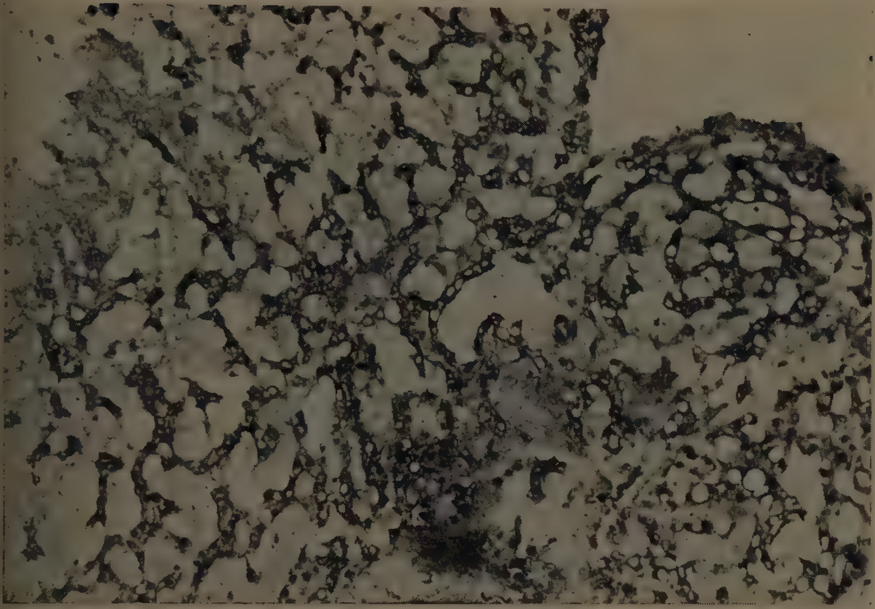

FIGURE 24. Aspirate from sacrum showing chordoma. Clot. ×140.

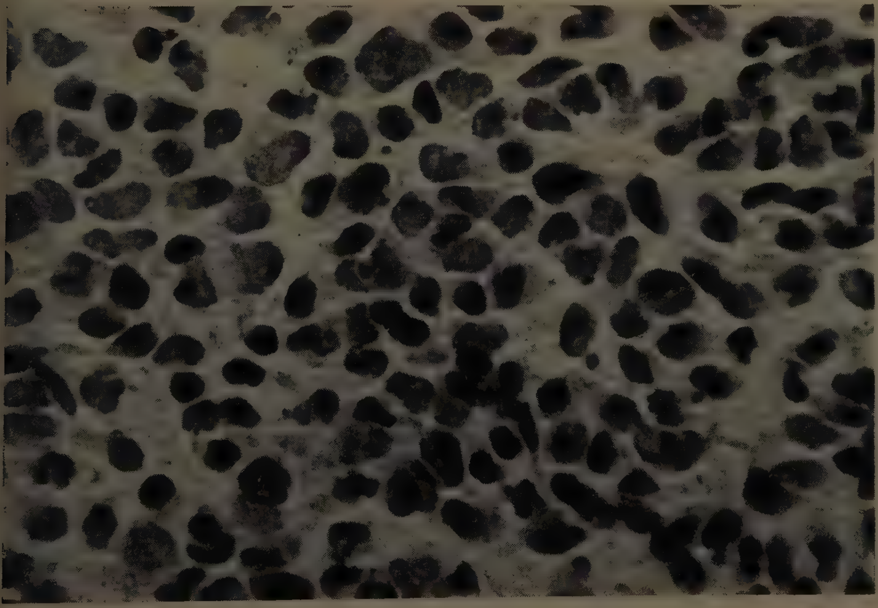

FIGURE 25. Aspirate from a soft part sarcoma classified as a myosarcoma. Smear. ×310.

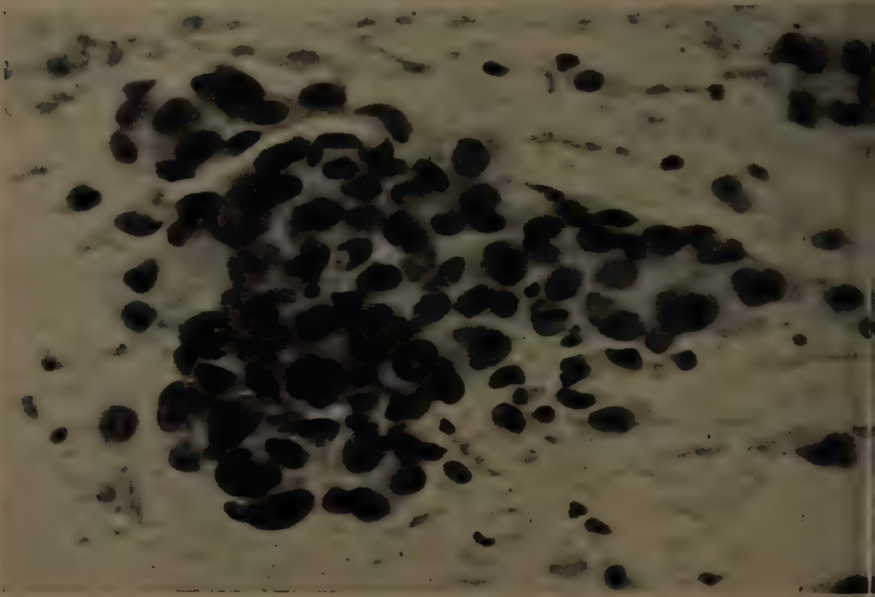

FIGURE 26. Aspirate from a lung lesion showing carcinoma. Smear. ×310.

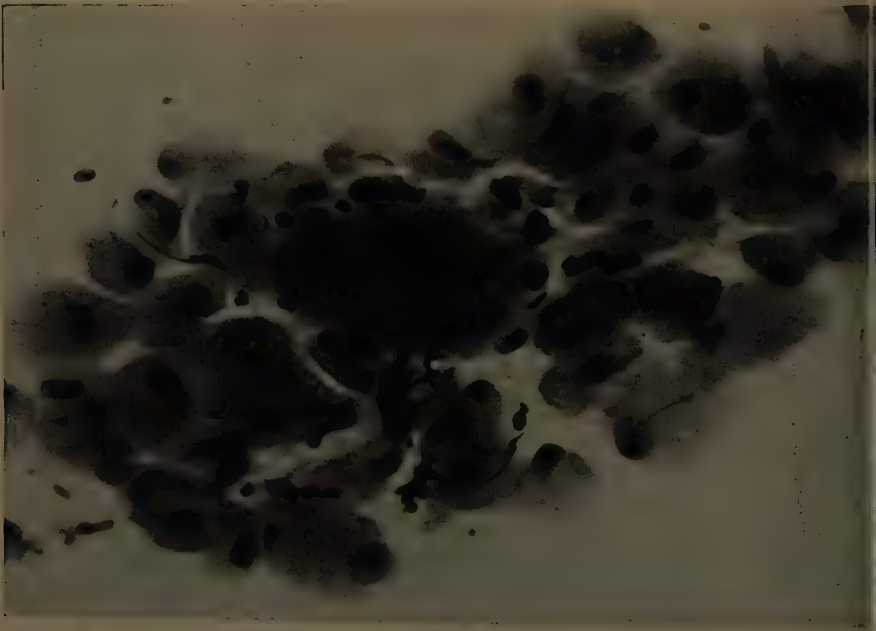

FIGURE 27. Aspirate from the posterior mediastinum showing cells of a ganglioneuroma. Smear. ×310.

URE 25), fibrosarcoma,[57] lipoma, and other mesenchymal lesions. Most frequently, the diagnosis of malignant tumors is about as far as one can go, although the clot occasionally helps to clarify the histogenesis.

Thorax, Lung, and Mediastinum

On the Thoracic Service,[61] the preferable lung lesion for aspiration is a discrete peripheral one (FIGURE 26). The procedure is indicated when five sputa or washings fail to reveal cancer cells and when exploratory thoracotomy is contraindicated by associated severe systemic disease, advanced age, or patient refusal, and there are no other sites available from which to establish the diagnosis. Emphysema is a contraindication, especially with blebs and bullae, because of the possibility of producing a tension pneumothorax. There is danger in aspirating lesions adjacent to major vessels, as in the apex near the subclavian vessels or at the pulmonary root.

The procedure is applicable in lesions of the posterior mediastinum (FIGURE 27), especially those in the gutter, when surgery is contraindicated or refused by the patient. The possibility of the presence of an anterior meningocele should be kept in mind, although aspiration would not be dangerous unless infection supervened.

Lesions of the anterior mediastinum suitable for aspiration are those in which the tumor is against the chest wall, and in which chest fluoroscopy and/or angiocardiography definitely show the lesion to be separate from the heart and aorta, also those lesions located away from the superior *vena cava*, either more lateral or inferior, on the right, and those that can be approached through the left anterior chest.

Ribs and vertebrae may be aspirated when one can keep away from the aorta.

The major complications are air embolisms, which can be prevented by positioning the patient with head down, and pneumothorax, either due to air being allowed inadvertently to enter through the aspirating needle or from puncture of blebs, bullae, or pneumatocele. Rarely have deaths been reported following aspiration of the thorax. Spread of tuberculous lesions, empyema, or seeding of the pleura by cancer cells are theoretical possibilities, but have not been observed. Although no seeding of aspiration tracks has been observed, growth along a drainage tube and in thoracotomy scars have been seen rarely. Minor hemoptysis may occur.

Occasionally, when the lung is exposed at operation, the lesion may be aspirated for diagnosis before definitive surgery is performed. Several authors have recorded their experience with aspiration biopsy of lung lesions.[3, 10, 11, 12, 28]

Genitourinary

The Genitourinary Service[76] employs aspiration for renal, prostatic, and metastatic lesions. Usually aspiration of kidney or prostatic tumors is performed when no attempt at operative excision is planned, but where pathologic diagnosis is needed.

Aspiration has not been employed in testicular tumors. Aspiration is most useful in establishing a pathologic diagnosis in cases of clinically advanced

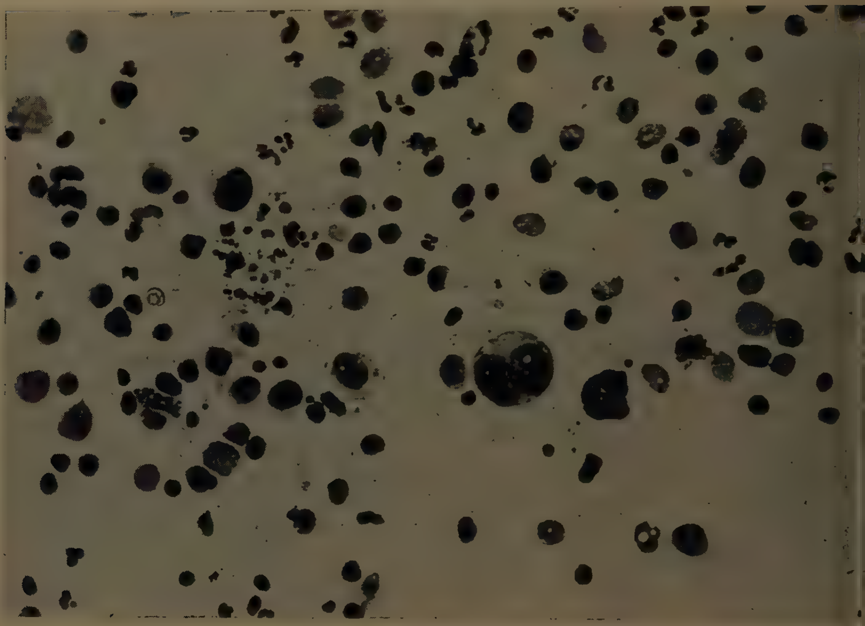

FIGURE 28. Aspirate from Hodgkin's disease showing a Reed-Sternberg cell and eosinophils. Smear. ×31

prostatic cancer. In a recent report by Kaufman *et al.*,[38] needle biopsy ha been found useful in the diagnosis of early prostatic carcinoma. Clarke *et al.* have reported one case in which an implant grew in the perineum 13 month following needle biopsy. Spring and Alden[69] have recorded their observation in a series of 83 cases in which needle biopsy was performed. Renal aspira tion[34, 40, 58] has been employed in studying various conditions of the kidney

Lymphoma

The malignant lymphomas present difficulty in diagnosis in aspirated ma terial and, most often, a node excision is required. Of the various lymphomas Hodgkin's disease (FIGURE 28) and reticulum cell sarcoma (FIGURE 29) are th more readily diagnosed. The lymphomas composed of small cells are not easily diagnosed on aspiration.

Morrison *et al.*,[56] Pavlovsky,[59, 60] Strunge,[71] Tempka and Kubiczek,[72] Dear ing,[17] and others have used aspiration biopsy in the study of various lymp node lesions. Loeske,[42] in a small series of aspirations in Hodgkin's disease obtained 56 per cent positive results.

Liver, Spleen, and Pancreas

Occasionally aspirations of the spleen are employed, and Gaucher's, Hodgkin's, and other diseases may be diagnosed. Splenic aspirations, however are performed infrequently. Morrison *et al.*[55] have reported upon their study

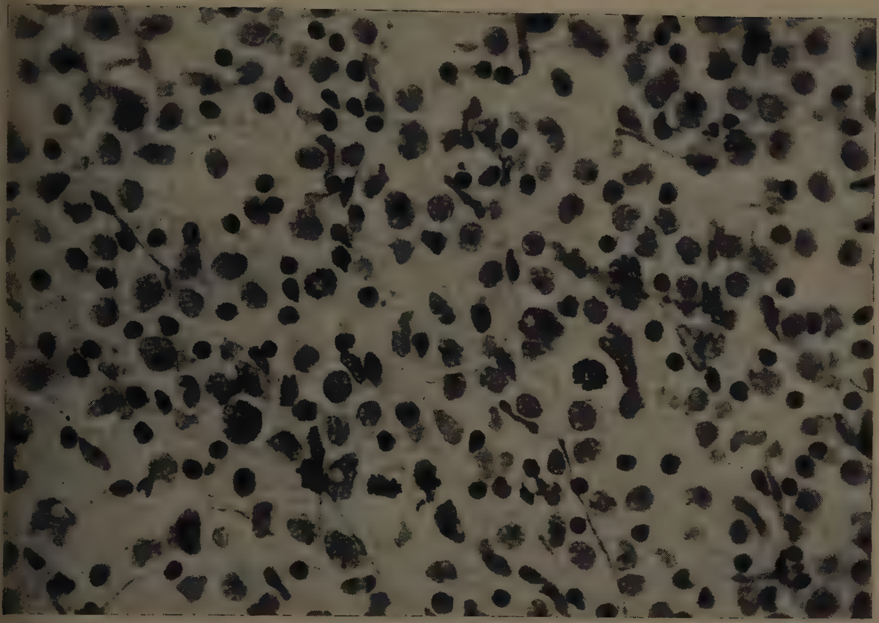

FIGURE 29. Aspirate from a cervical lymph node showing reticulum cell sarcoma. Smear. ×310.

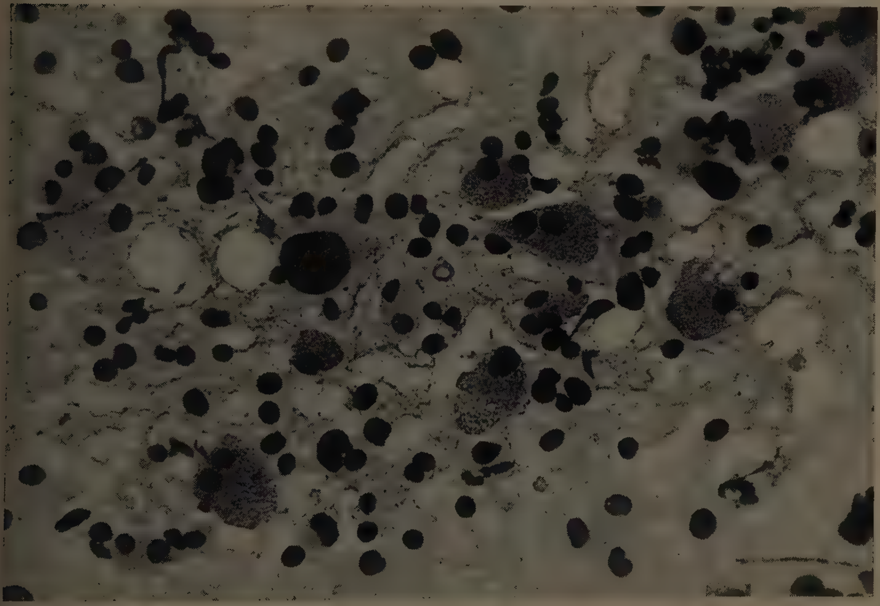

FIGURE 30. Aspirate from liver showing normal and malignant cells of a hepatoma Smear. ×310.

of splenic aspiration. Moeschlin[53] has published a monograph on the subject and Shapiro and Watson[63] have recorded their observations of splenic aspiration in multiple myeloma.

Aspiration of the liver (FIGURE 30), as such, is also infrequently performed. Ordinarily, the procedure employed is the standard liver needle biopsy with a Silverman needle. In view of the extensive reports on liver needle biopsy, no further comment will be made.

The spleen, liver, and other viscera, such as the pancreas,[39] may be aspirated during operative procedures, since this is a quick and easy way of obtaining a small amount of material without cutting into the lesion or organ before a definitive plan of operation is formulated. Mayo and Baskin[52] have found needle biopsy useful in establishing the diagnosis of solitary liver nodules during abdominal surgery.

Miscellaneous

Aspiration biopsy may be performed in the central nervous system and, in reality, many of the specimens received from the operating neurosurgeon amount to this since, many times, the minute specimens must be smeared,[54] since sectioning is not possible. Smears from thoracic and abdominal fluids (FIGURE 31) are frequently made, but may offer much difficulty in interpretation because of the atypical appearance and arrangement of mesothelial cells. Foot[23, 24] and Luse and Reagan[44, 45] have recorded their observations on cytological studies of pleural fluids.

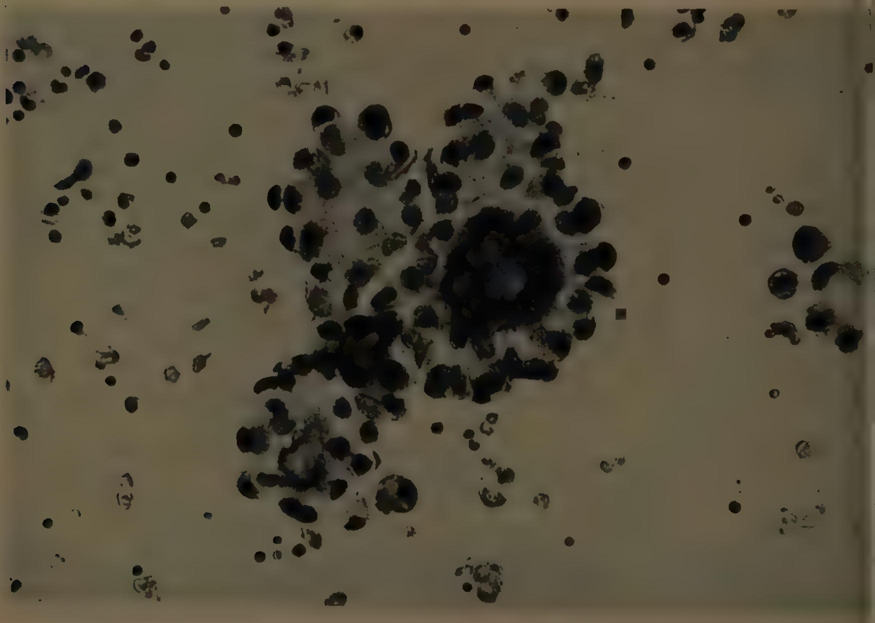

FIGURE 31. Ascitic fluid showing a papillation of an ovarian carcinoma. Note psammoma bodies. Smear. ×310.

Summary and Conclusions

The technique and application of aspiration biopsy as performed at Memorial Center has been reviewed in view of 20-odd years' experience.

During this period, it has become a routine procedure on several services as an aid in diagnosis and management of cancer and other diseases. It has not been found to affect the course of a disease unfavorably.

It is hoped that this brief report will stimulate those physicians in a suitable environment to employ the procedure in the diagnosis of neoplastic diseases.

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CYTOLOGY OF PULMONARY CARCINOMA

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Cytology in the diagnosis of cancer of the respiratory system is not new, for isolated reports dealing with neoplastic cells in sputum have been recorded almost as early as a century ago.[1] In 1860, Beale noted single and clumped tumor cells in unstained smears of sputum from a patient with carcinoma of the pharynx. In 1876, Hampeln identified neoplastic cells in the sputum of a patient whose extremity had been amputated sometime previously for sarcoma. In 1890, Huber isolated tumor cells in sputum from a case of sarcoma of the lungs. In 1895, Betschart demonstrated the superiority of stained over unstained smears of sputum in cases of pulmonary tumors.

While these early efforts were all directed toward the identification of neoplastic cells in smears of sputum, Bahrenberg, in 1895, and Mandlebaum, in 1900, each described a method of examining sputum and other fluids for tumor cells by first sedimenting the formed elements and then treating the sediment as a piece of tissue. Because this method followed the conventional histologic procedure used by pathologists, and because it yielded good results in connection with serous fluids, it was universally adopted as a routine technique for over a quarter of a century. Its adaptation to sputum, however, yielded mixed results. Thus, in England, positive results were recorded in as high as 68 per cent of cases of carcinoma of the lung; in Russia, in as high as 70 per cent; and, in Denmark, in as high as 86 per cent.[2] Contrariwise, most leading clinics in the United States found the method of little or no value.[2] It was for this reason that, over a decade ago, we directed our attention to the examination, not of sputum, but of the more concentrated bronchoscopically removed secretions, and not by the regular paraffin-block technique but by the more precise Papanicolaou method. Our preliminary results were published in 1946.[2] Since then, with more concentrated effort, most bronchoscopic and chest clinics in the United States are using this or a modified procedure with considerable success.

Materials and Method

In carcinoma of the lung, there are four materials that are suited for cytologic studies: sputum, bronchoscopically removed secretions, saline washings, and pleural fluid. Sputum is obtained with the greatest ease and with the least discomfort to the patient. It is, however, not nearly as fruitful as bronchoscopically removed secretions or as saline washings because, in the early stages of pulmonary carcinoma (when it is mandatory to make the diagnosis to effect a cure), there is little or no sputum and, when sputum does appear, neoplastic cells are difficult to find in the concomitantly present, overabundant menstruum. Although bronchoscopically removed secretions are considerably more concentrated, they also may be objected to on the same grounds. Saline washings, however, appear to be ideal if the procedure of obtaining them is performed with care. Initially, the tumor is precisely localized roentgenographically. If roentgenograms are normal, the neoplasm can be located bronchoscopically by following the trail of mucoid, purulent, or hemorrhagic secretion for which the patient is examined. In either case, a bronchoscopy is performed in the usual manner, using a curved (for the upper lobes) and a straight (for the lower lobes) flexible-tipped bronchoscopic aspirator. The material in the trachea and main bronchi is aspirated and discarded, or is bypassed. The tip of the aspirator is inserted into the bronchus draining the suspected tumor site and, under direct vision, from 2 to 5 cc. of normal saline are gingerly instilled. The amount of saline used should be enough only to insure a mucoid or bloody return. If the return is watery, too much saline has been used and the results are uniformly disappointing. The washings thus obtainable are then collected in a test tube that is attached directly to a side arm of the collector just distal to the attachment of the suction tube. In the laboratory, the collected material is poured into a Petri dish, and Papanicolaou smears are prepared of areas that are most likely to contain neoplastic cells, that is, of small gray particles or blood-streaked areas. Pleural fluid, as a source of material for the diagnosis of carcinoma of the lung, is obtainable only in the hopeless cases, and is thus of diagnostic but not of curative importance. It is secured by thoracentesis, and smears are made of the buffy layer overriding the darker part of the sediment.

Interpretation of Smears

Smears of any material examined contain normal cells, degenerating cells, inflammatory cells, neoplastic cells, fibrin, debris, and other elements. Obviously, it is important to become familiar with each of these in order that they may not be confused with cancer cells. Since detailed descriptions of the appearances of the various elements have been given in several other publications,[1, 2, 3] they need not be repeated here. It is sufficient to state that cancer cells are identified by the same deviations from normal of shape, size, and staining qualities used in classifying the histologic sections. Apparently, most mistakes are made in interpreting cells or clumps of cells that are not clearly visualized. Unless the cellular outline, the cytoplasm, and the nucleus are clearly visible, the cells in question should be completely ignored.

Results

Based on our experience with over 1,000 cases of proved carcinoma of the lung, a positive cytologic diagnosis should be possible in about 85 per cent of all cases. A positive bronchoscopic biopsy can be expected in about one third of all cases, indirect bronchoscopic evidence of tumor (in the form of bronchial stenosis, fixation, *etc.*) in slightly more than one third of all cases, and completely negative bronchoscopy but positive cytologic findings in about one fourth of all cases. It is the last-mentioned group that offers the greatest hope from a curative standpoint for, in an analysis of consecutive pneumonectomies for carcinoma, we have found that only 8 per cent of patients with a negative bronchoscopic examination have metastasis to bronchial and mediasstinal lymph nodes, while over 50 per cent of patients showing any bronchoscopic evidence of tumor have peribronchial and mediastinal spread of tumor.

Overdiagnosis

In our series of over 1,000 cases, we made an overdiagnosis of carcinoma in four patients in whom a pneumonectomy was subsequently performed. The first case was one of multiple unilateral abscesses; the second, of idiopathic atelectasis of an upper lobe in a patient who had undergone a mastectomy for carcinoma a year previously; the third, of atelectasis consequent to obstruction by an aspirated bean; and the fourth, of massive "unilateral" anthracosilicosis. In each of these instances, a positive cytologic diagnosis was not warranted. It was made after repeated examinations and upon pressure from the clinician. In other words, it resulted from an attempt to "stretch" the usual criteria.

Sources of Error

The usual sources of error may be listed as follows: (1) improper bronchoscopic collection of material, including use of excessive amounts of saline; (2) improper preparation of smears in the laboratory, including haphazard selection of material to be smeared, thick smears, overstaining, *etc.*; (3) "stretching" the well-established criteria in an effort to make a diagnosis early in the course of the disease; and (4) being "influenced" or "pressured" by the clinician.

Conclusions

When performed with care, the cytologic diagnosis of carcinoma of the lung made from an examination of bronchial secretions is now on as firm a basis as is the histologic diagnosis made from bronchoscopically or surgically removed tissue. The method is highly recommended.

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NEWER ADVANCES IN THE EXFOLIATIVE CYTOLOGY OF THE GASTROINTESTINAL TRACT*

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Recent studies have established exfoliative cytology as a valuable method for the diagnosis of gastrointestinal cancer.[1, 2, 3] This communication concerns itself primarily with the newer advances in diagnosis and research that have resulted from the use of this technique. The literature in this field has been the subject of recent communications[3, 4, 5] and will not be extensively reviewed.

The opinions expressed in this paper represent the personal experiences of the author and his interpretations of the work of others. An attempt has been made to evaluate this field objectively. Many of the conclusions, of necessity, are based solely upon clinical experience rather than upon the more controlled observations of the laboratory.

The recent increase in diagnostic accuracy of gastrointestinal exfoliative cytology is attributable to increasing experience in interpretation of cellular morphology and to better methods of collecting freshly exfoliated cells before they become digested.

Initially, these techniques were used solely for diagnosing cancer in the relatively inaccessible portions of the alimentary tract. Cytologic examinations may be repeated frequently with little discomfort to the patient, and it has become evident that this technique may be used also to study the evolution of various types of gastrointestinal disease.

Esophagus

Esophageal cytologic examination should be used more often because of the reliability and simplicity of the method. The speed with which barium traverses the esophagus and the inaccessability of this organ to manual palpation limits the usefulness of X ray in the diagnosis of small early lesions. Neoplastic disease is frequently located in the lower end of the esophagus,[6] an area that may be difficult to visualize by esophagoscopy. This is one of the limitations of the latter procedure. Morever, it is well known that a negative esophagoscopic biopsy does not preclude carcinoma. Preoperative cytologic diagnoses of esophageal carcinoma have been confirmed repeatedly in spite of negative radiologic or esophagoscopic studies.[7 to 16] The combined use of cytology, esophagoscopy, biopsy, and radiography should result in earlier and more accurate diagnoses of potentionally curable lesions.

Cell collection methods. Various abrasive and other complicated techniques have been proposed for obtaining cells from this organ. In this laboratory, the following harmless routine has proved effective: if no obstruction is encountered, a Levin tube is passed into the stomach with the patient in the

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* This project was supported by funds accruing to Initiative 171 of the State of Washington for Research in Biology and Medicine, by a research grant from the Damon Runyon Memorial Fund for Cancer Research, Inc., New York, N. Y., and by the Washington State Division of the American Cancer Society.

seated position. The gastric contents are evacuated and discarded unless tissue particles are present. The patient then drinks 100 cc. of Ringer's solution, which washes the whole length of the esophagus. This material is immediately aspirated as it reaches the stomach. Lavage is then performed via the Levin tube with 100 cc. quantities of Ringer's solution at four separate esophageal levels, with the patient in the supine position. If an area of resistance is met, especially vigorous lavage is then performed at this level. The lavage solution must be processed without delay. It is centrifuged, the sediment is smeared on slides, and the smears are fixed while wet.

Malignant cells may be obtained when the tube will not pass a stenotic lesion. In such cases, it may be desirable to induce mild regurgitation. The fauces may be stimulated by trying to pass the tube beyond the obstruction so that it coils up slightly in the pharynx. Fluid should be aspirated as the patient gags, as cells may be regurgitated from the distal constricting lesion.

Some workers obtain cells by direct suction during esophagoscopy. This method samples a small area. In the author's experience, confusing cellular atypia is more frequent after this type of manipulation. Furthermore, any procedure requiring esophagoscopy is too complicated for frequent use.

Morphologic cytology. The usual nuclear aberrations[2, 7, 17, 18] are observed in malignant esophageal squamous cells (FIGURE 1). The morphology of exfoliated esophageal epithelium has not been extensively studied, but much has been learned by analogy from the squamous morphology of the cervix and bronchi. In our present state of knowledge, esophageal cytology seems simpler than that of the latter two organs. This, however, may be an impression that will change with further study.

The cytologic appearance often permits differentiation of squamous from adenocarcinoma. Cytoplasmic vacuolization suggesting mucus formation favors adenocarcinoma which, in turn, strongly suggests that the tumor originates in the stomach.[19] The more anaplastic squamous carcinomas may be indistinguishable from adenocarcinoma cytologically. The diagnosis of

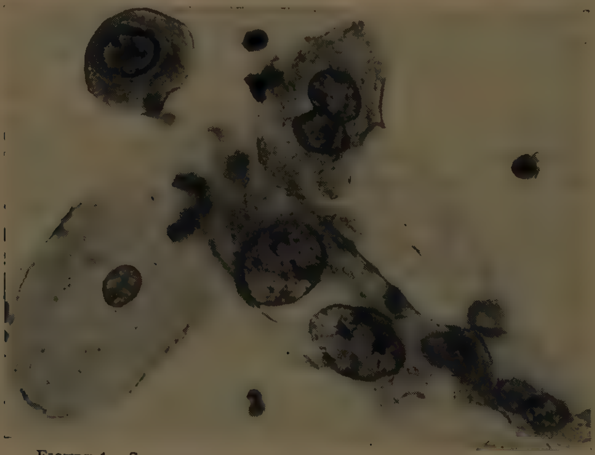


FIGURE 1. Squamous carcinoma. Benign cell (lower left). ×750.

highly keratinized squamous carcinoma is not difficult. The presence of "malignant pearls" is pathognomonic of squamous origin. Various bizarre squamous forms such as "tadpole" and "fiber" cells are also helpful.

Atypical cells, which are easily confused with carcinoma, may be exfoliated in esophagitis[11, 15] and peptic ulcer[20, 21] of the esophagus. Currently, there are no infallible criteria that help differentiate these puzzling cells from malignant ones. Perhaps they will be developed with further experience. Fortunately, this confusing atypia is rare. Various sources of these puzzling cells have been suggested, such as: atypical epithelium that has been exposed by sloughing of the superficial layers;[15] the squamous cells from metaplastic esophageal glands;[15] and the young fibroblasts or endothelial cells originating in exuberant granulation tissue.[11] If peptic ulcer or esophagitis is suspected and some questionably malignant cells are found, repreated examinations are mandatory, preferably after treatment. It is unwise to diagnose cancer in such instances on the basis of a few cells, present in only one examination.

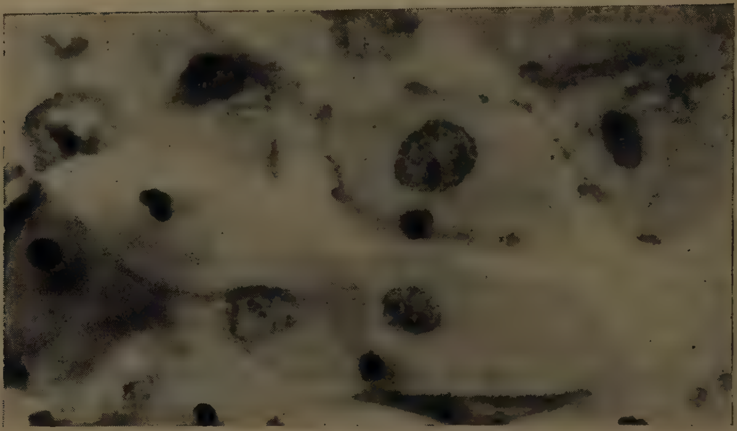
Graham and Rheault[22] noted cytoplasmic and nuclear enlargement, multinucleation, and abnormal chromatin distribution in some of the swallowed squamous epithelium found in the stomachs of patients with *untreated* pernicious anemia. This macrocytosis is not confined to the mouth, pharynx, and esophagus, but has been observed at other exfoliating surfaces.[22] These findings have been confirmed in this and other laboratories.[23] They have also been noted in two cases of probable folic acid deficiency (megaloblastic anemia of pregnancy and nutritional macrocytic anemia)[24, 25] (FIGURES 2 and 3). This macrocytosis reverts towards normal with B_{12}[22, 23] or folic acid therapy.[24, 25] Cytologic abnormalities have also been noted histologically in human beings[26, 27] and in experimental animals[28-33] receiving folic acid antagonists.

From a practical point of view, it seems best to defer a diagnosis of carcinoma based on a few malignant-appearing squamous cells in patients with nutritional deficiency or macrocytic anemia. Re-examination after specific parenteral treatment should confirm or disprove the diagnosis. The parenteral route is preferred to the oral one in order to avoid errors due to defective intestinal absorption.

Squamous macrocytosis and nuclear atypia similar to that described above may be seen in some of the benign squamous cells that accompany some esophageal carcinomas or that may be present after radiation therapy.[23] The reason for these cellular changes is poorly understood. They may be similar to the radiomimetic changes that Dustin[30] has observed in the mouse after antifolics.

Indications. Cytologic examination of the esophagus should be freely employed in patients who have any difficulty in swallowing. The ease of esophageal lavage makes it an office procedure that may be used in the patient with minimal symptoms. The distal esophagus is especially inaccessible to the usual diagnostic methods. The cytologic method has proved itself in this area.[7, 11] It is diagnostically helpful in stricture or narrowing of the esophagus from any cause, and is indicated when a malignancy is suspected or recurrence is under consideration.

Cardiospasm,[14, 34-36] sideropenic dysphagia[37 to 40] (Plummer-Vinson syn-



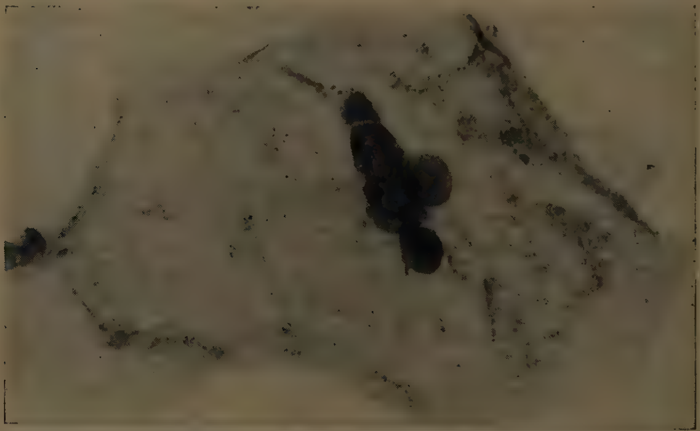


FIGURE 2 (upper). Squamous cells (nuclear enlargement and hyperchromatism). ×750.
FIGURE 3 (lower). Enlarged, multinucleated squamous cell (untreated folic acid deficiency). ×750.

drome), and long-standing stricture[41] all show a tendency to undergo carcinomatous degeneration. Klayman[14] has shown the value of cytology in detecting carcinomas complicating cardiospasm. Similarly, Andersen *et al.*[7] have discovered malignant changes in the cells shed from an old lye stricture. Cytologic screening of these patients at regular intervals may reveal unsuspected tumors.

Accuracy. The best results are reported by those investigators who control both collection and interpretation of the cytologic material. In the hands of these workers, 54 of 56 carcinomas of the esophagus were correctly diagnosed by exfoliative cytology prior to operation.[2, 14, 16] In reviewing the depressing results of surgical therapy in this disease, it is well to remember that *in situ* carcinoma has been diagnosed by this method.[8] One third of squamous carcinomas of the esophagus are theoretically "curable" after symptoms have developed.[42] It is possible that more tumors would be curable in reality if the diagnosis were made earlier in the asymptomatic phase.

Future research. There are several lines of esophageal research that suggest themselves:

The role of leukoplakia as a cancer precursor in the esophagus bears careful investigation in view of its known tendency to carcinomatous change in other organs that have areas of sqaumous epithelium. Serial cytologic studies controlled by esophagoscopic biopsies should prove informative. The presence of severe leukoplakia in one of Klayman's[14] cases of cardiospasm with cancer is of interest. Suzman[43] has reported a post-mortem examination in a patient with sideropenic dysphagia. Portions of this patient's esophagus had a leukoplakic appearance, although no cancer was present. Oral leukoplakia also has been observed in sideropenic patients who developed hypopharyngeal and/or esophageal cancer.[40]

Messelt[13] noted the predominance of highly cornified superficial squamous cells in a hypopharyngeal smear of a patient with sideropenic dysphagia. It is of interest that there is an increased incidence of keratinized, well-differentiated squamous carcinoma of the mouth, hypopharynx, and esophagus in the latter disease,[38, 40] which should be particularly susceptible to serial cytologic study. The clinical belief that this condition is a reversible iron deficiency is of interest. How does the cytology and histology of the upper gastrointestinal tract change with iron and dietary therapy? Why do certain patients go on to develop cancer despite treatment?[40]

Esophageal lavage may prove to be a useful method for studying the epithelial macrocytosis of folic acid deficiency whatever its cause (malnutrition, malabsorption, or antifolics). There are those who believe that the pterdines are among the most important growth-accelerating and growth-determining agents known to man.[44] Exfoliative cytology may prove valuable in the investigation of the effects of these substances.

Buschke,[45] after surveying a large number of cases treated by surgery or radiotherapy, concluded that carcinomas of the upper fourth of the esophagus responded best to X ray, and that those of the lower quarter responded best to surgery. Cancer of the midthoracic esophagus responded in parallel fashion to either form of treatment. Graham and Graham[46] have described certain morphologic and tinctorial alterations in benign basal cells of the vaginal smear which they designate the *sensitization response* or "S.R." Patients with cervical carcinoma who have many S.R. cells in their vaginal smears will respond more favorably to radiotherapy than to surgical excision. It might be worth accessing the prognostic value of S.R. changes in the basal squamous cells of the esophagus. This might offer a rational basis for choice of treatment in midthoracic carcinoma.

Stomach

Contamination of the gastric contents with food or other detritus and digestion of the exfoliated gastric cells are the main obstacles to accurate cytologic diagnosis. Careful preparation of the patient helps ensure an empty stomach. An overnight fast is the usual preparation for examination. More vigorous measures are indicated in the presence of obstruction: liquid diet for one or two days, and lavage until clear with a large lumen tube the night before examination. Cellular digestion can be minimized by several new collection techniques.

Cell collection methods. In many gastric malignancies, any collection method will yield diagnostic cells. A superior technique will procure diagnostic cells in the difficult case, despite anatomic inaccessability or minimal exfoliation. The real test of a cell-collection method is the percentage of cancers that are missed. Naturally, the same technique in the hands of different workers will not yield comparable results. Experienced, meticulous technicians overlook few cancers.

Simple saline lavage, carefully performed, has yielded a high accuracy in the hands of a few investigators.[47, 48] This method may be used by a technician with safety and little discomfort to the patient. Theoretically, saline lavage samples the entire mucosal surface of the stomach. On the other hand, the number of cells obtained and their preservation does not compare with some of the more complicated techniques. This may account in part for the lower accuracy of this method reported by other workers.[49, 50]

The abrasive balloon[51] may yield well-preserved sheets of cells. It samples most of the mucosal surface, but it may miss a constricting lesion of the distal stomach. This technique is sufficiently complicated to require a physician operator, although some expert technicians have obtained admirable results. The examination is uncomfortable for the patient and somewhat time-consuming.[52] The antral abrasive balloon[2] is similar to the balloon of Panico *et al.*[51] but, theoretically, it abrades the distal stomach.

Various other rapid abrasive techniques have been suggested. All the methods described to date have one disadvantage in common: they do not sample the *entire* mucosa and, therefore, avoidable false-negative examinations are inevitable. At the turn of the century, Hemmeter[53] used a rubber tube as a blind curet. The detached pieces of gastric mucosa were then recovered by lavage. This method did not gain wide acceptance, because it was considered too traumatic. Gladstone[54] suggested the passage of a Levin tube with numerous sponges attached to its surface. Henning and Witte[55] described a "cell tampon." This instrument consists of a soft rubber tube that contains a rubber sponge attached to a mandrin. When the tube is in proper position, the sponge is pushed forward and brushed against the gastric wall. This method has its highest accuracy in the cardia, and is least reliable in tumors of the antrum.[55] Ayre and Oren[56] suggested the use of a similar instrument employing a rotating brush. Zamcheck[57] has commented on the potential danger of the latter instrument: "The apparatus is crude at the present time. The end of the tube is made of metal and is sharp. Without fluoroscopy the operators have no real measure of the position of the brush in the stomach or when the brush may snag an area of mucosa which when twisted can be of considerable size. Furthermore, when the brushes are ensheathed, a nearby area of mucosa can get caught in the closing brushes and be traumatically torn when the instrument is withdrawn." There is no doubt that the Ayre brush abrades beautiful cellular material from a limited area. Its accuracy in diagnosing gastric cancer is disappointing.[58]

Rosenthal and Traut[59] developed a clever method for releasing undigested cells located in the mucous-barrier covering the gastric mucosa, by using papain as a mucolytic enzyme. At times, the diagnostic cells obtained by this technique are excellent and, on other occasions, they are badly digested. The degree of digestion is unpredictable because papain powder is a crude mixture of enzymes that loses its activity with time at a rate dependent on many uncontrollable variables.[60]

Crystallized chymotrypsin* in a suitable buffer has been employed for gastric lavage in this laboratory with very promising results.[61] Chymotrypsin's mode of action is similar to papain. It liquifies the mucus that lines the stomach, and releases freshly exfoliated cells without changing their morphology. In the fasting state, the stomach is lavaged via a Levin tube with Ringer's solution until the return is clear. The chymotrypsin in then introduced and left in the stomach for 10 minutes while the patient is positioned to insure contact with the whole mucosal surface. The recovered lavage solution should be refrigerated until there is sufficient volume for centrifugation. The sediment must be smeared and fixed without delay. The procedure may be performed by a technician, and is minimally discomforting to the patient. It is somewhat time-consuming, and may take from one half to one hour to perform. Abundant sheets and single undigested cells are obtained. Klayman *et al.*[62] have tested this procedure in a series of cases with satisfactory results. In the author's opinion, this is the most practical method currently available.

Morphologic cytology. Many of the cells in gastric smears do not originate in the stomach. Squamous epithelium from the mouth, nose, pharynx, and esophagus may predominate. Leukocytes, histiocytes, and respiratory epithelium may be seen. In the achlorhydric stomach, fungus and bacteria may be evident.

The benign and malignant morphology of gastric columnar epithelium is thoroughly reviewed elsewhere.[2, 17, 18] This section will concern itself with recent work on the gastric columnar cytology of B_{12} and folic acid deficiency, and the characteristic changes in *treated* pernicious anemia. The cytology of malignant lymphoma, primary in the stomach, will be described.

In *untreated* pernicious anemia, Graham and Rheault[22] described macrocytosis and nuclear abnormalities in cells exfoliated from the esophagus, lung, vagina, and stomach. The most obvious change was an increase in size of both nucleus and cytoplasm. Multinucleation was seen in some of the enlarged cells. The nuclear chromatin, frequently, was distributed unevenly in clumps, giving an appearance of rapid growth. These findings have been confirmed by others,[23] and have been observed by the author in two instances of probable folic acid deficiency[24, 25] (FIGURE 4).

Some recent experimental work may have a bearing on this large cell phenomenon. Hughes[63, 64] has observed chick-tissue cultures by phase-contrast cine-micrography after Aminopterin treatment. Spindle formation was inhibited, and mitosis was arrested in metaphase. Occasionally, the chromo-

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* Available from Armour Laboratories, Kankakee, Ill.

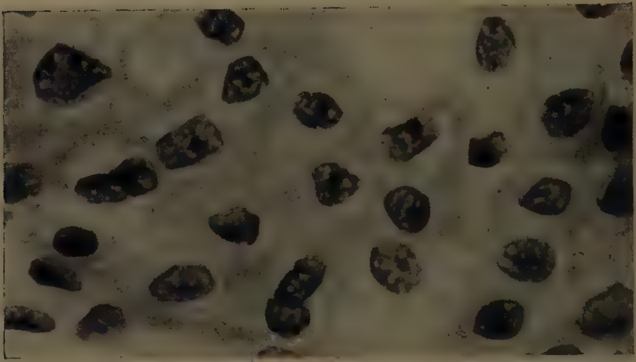
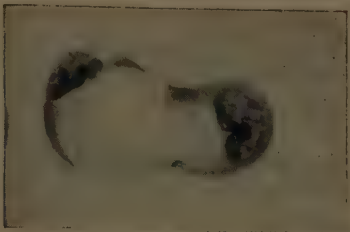


FIGURE 4. Enlarged gastric columnar cells (untreated folic acid deficiency). ×750.

somes reconstructed into an enlarged tetraploid nucleus within the undivided cell. Jacobson has observed cellular enlargement in the intestinal epithelium of mice that had been treated with Aminopterin *in vivo*.[31, 32] The latter worker has shown that the arrest of cell division caused by Aminopterin cannot be prevented by folic acid given prior to or simultaneously with Aminopterin. On the other hand, *Leuconostoc citrovorum* factor in excess, when applied simultaneously with Aminopterin, prevents the metaphase arrest of dividing cells.[32, 65]

Specific treatment of patients with B_{12} or folic acid deficiency reverses the macrocytosis of exfoliated epithelial cells.[22, 23, 24, 25] There is one exception in *treated* pernicious anemia. Large gastric columnar cells remain, they are unaffected by years of vitamin B_{12} or liver therapy, and they average twice the normal size.[66] The reason that these large cells persist is not known. It will be important to ascertain if these large nuclei are tetraploid. It is possible that they are related to the irreversible atrophy of the gastric epithelium that is almost universal in pernicious anemia. Reisner *et al.*[67] and Mollin *et al.*[68] suggested that this permanent gastric atrophy may be caused by a long subclinical period of B_{12} deficiency. One might speculate that gastric macrocytosis, which is unaffected by B_{12}, reflects an irreversible metabolic adaptation.

The malignant appearance of some of the gastric cells in *untreated* folic acid and B_{12} deficiency is of both theoretical interest and practical importance. FIGURE 5 shows malignant-appearing signet-ring cells found in a woman with megaloblastic anemia of pregnancy, one week *post partum*. Malignant-appear-



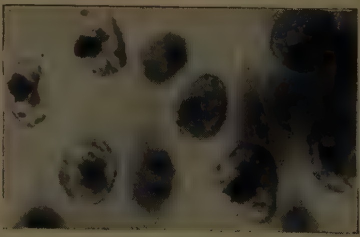


FIGURE 5 (left). Signet cells (megaloblastic anemia of pregnancy). ×750.
FIGURE 6 (right). Active large cells (nutritional macrocytic anemia). ×750

ing gastric cells have also been observed in a man with nutritional macrocytic anemia prior to treatment[25] (FIGURE 6). These two patients had probable folic acid deficiency. Their abnormal gastric cytology reverted to normal with treatment. Although large gastric cells are present before and after treatment in pernicious anemia, those present prior to therapy may have a more bizarre appearance. In this group of cases, it has proved best to defer diagnoses of cancer until treatment has caused the bizarre atypias to disappear.

The majority of gastric columnar cells exfoliated in *treated* pernicious anemia are normal in every way. In all patients examined in this laboratory, at least a few groups of large columnar cells have been found that differ characteristically from the normal. Although these cells are regularly observed in pernicious anemia, they are not pathognomonic. They have been found in some patients with histamine fast achlorhydria and no evidence of pernicious anemia,[23] in intestinalized atrophic gastritis,[25] and in certain gastric carcinomas.[23] FIGURE 7 compares the normal and large cells.

The exact gastric source of these cells remains to be determined. Their significance is puzzling and merits further study. This large columnar cell has a distinctive nuclear appearance.[66] The nuclear membrane is delicate, and may be typically folded or creased. The nucleoli are either small or inapparent. The chromatin is distributed in small aggregates that stand out on the relatively empty background of the nucleus. The cytoplasm of the large cell is enlarged commensurate to the size of its nucleus, thus maintaining a normal nuclear-to-cytoplasmic ratio. The variable cytoplasmic morphology may offer clues to the large cell's origin.[24] Most often, the cytoplasm contains no mucus and has an eosinophilic, finely-vacuolated, or granular appearance. A mucus containing theca of variable size may be present. Small amounts of apically located mucus makes some large cells resemble the mucous neck or surface cell of the stomach. At times, the mucus occupies most of the cytoplasm and pushes the large nucleus, basally producing a goblet cell appearance.

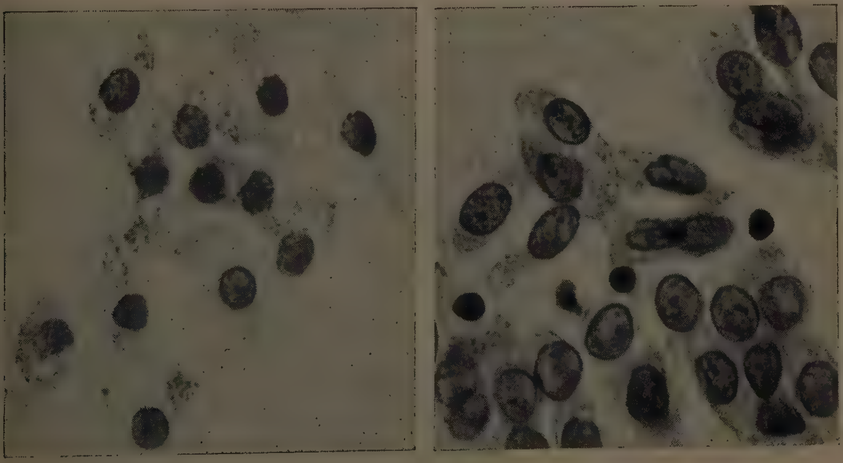


FIGURE 7. Treated pernicious anemia. (left) normal. ×750. (right) large. ×750.

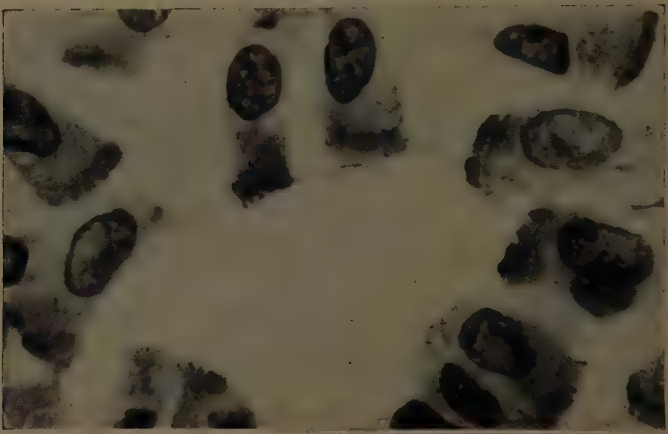


FIGURE 8. Intestinal absorptive cells (treated pernicious anemia). ×1800.

Exfoliated intestinal absorptive cells with normal-sized nuclei and striated borders are another indication of gastric intestinalization that is observed frequently in pernicious anemia[24] (FIGURE 8). Normal-sized goblet cells may also be seen.

Another type of large columnar cell is present in a smaller proportion of the patients with *treated* pernicious anemia. It is designated the active large cell.[24, 66] Characteristically, it has a heavy irregular nuclear membrane that is usually not folded. The nucleoli are large, and may be eosinophilic and refractile. The chromatin is increased in amount, and is distributed in coarse clumps (FIGURE 9). These active cells may be incorrectly interpreted as originating in a gastric carcinoma. On blind review of all the cases of *treated* pernicious anemia studied in this laboratory, active cells were consistently more frequent in patients who proved to have polyps on gastroscopic examination.[24]

In two cases of pernicious anemia who had developed cancer of the stomach, a wide morphologic spectrum was seen, from the large bland-appearing cell

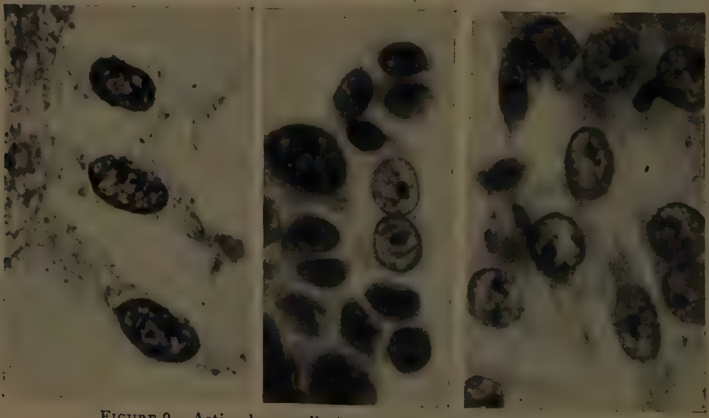


FIGURE 9. Active large cells (treated pernicious anemia). ×750.

through large gastric columnar cells of increasing activity to frank adenocarcinoma.[69] From a purely morphologic point of view, it would seem conceivable that the large cell, whether bland or active, may be the progenitor of the carcinoma. This fascinating problem merits intensive study.

Malignant lymphoma, localized to the stomach, is a diagnosis well worth making, because it differs from carcinoma in its tendency to remain localized,[70, 73] to respond to radiation,[70, 71, 74-81] and to have a better prognosis.[70, 77, 79, 82-85] X ray and gastroscopy may suggest the diagnosis, but they cannot make it definitively. A specific diagnosis may be made by exfoliative cytology. Papanicolaou's group was the first to make a preoperative diagnosis by this method.[50] The cytology of this condition has been described in detail recently.[86] The salient feature is the tendency for the cells in the smear to occur singly rather than in the clumps that are more characteristic of carcinoma. This solitary distribution is most striking.

Most commonly, the cells exfoliated by a gastric lymphoma are of one predominant type. This uniform picture is punctuated by rarer cells ranging from primitive stem cells to normally differentiated lymphocytes. The predominant cell is only slightly larger than a normal gastric cell. There is not the definite increased variation in nuclear size so typical of carcinoma. Because of this uniformity and relative lack of size increase, the diagnosis can be missed readily when using the low-power scanning lens (magnification ×100). Upon shifting to the high dry objective (magnification ×430), one may be surprised to find that many of the cells that one has ignored are actually malignant. The lymphoid nuclei characteristically have heavy nuclear membranes of smooth outline, closely packed chromatin aggregates, scant karyoplasm and nucleoli that, if visible, are less prominant than those seen in carcinoma. The lymphoid cytoplasm is usually sparse, forming a thin rim about the nucleus. At times, it is scarcely discernible. The finding of Sternberg-Reed cells has made the diagnosis of gastric involvement by Hodgkin's disease.[86] This type of lymphoma may also be confined to the stomach and is potentially curable.

The differential cytologic diagnosis between single-cell anaplastic carcinoma and lymphoma may be difficult. It is well to remember that a single malignant signet-ring cell sets the diagnosis of lymphoma in doubt.

Small cell lymphomas may be impossible to diagnose because the individual exfoliated cells show no clear malignant characteristics.[87] The diagnosis, in these cases, is primarily an architectural one, necessitating tissue examination. The cytologic diagnosis of gastric lymphoma is not possible if the tumor has not penetrated the mucosal surface sufficiently to exfoliate cells.

Indications. Cytologic examination of the stomach should be employed in selected cases. At present, technical complexities and lack of trained personnel prevent wider use of these methods in screening for cancer. If adequate facilities are available, selected screening is both feasible and desirable.

In differentiating benign from malignant gastric ulcer, it is not necessary to resort to laparotomy. The combined use of X ray, gastroscopy, and exfoliative cytology enables the clinician to make a correct preoperative diagnosis.[2, 3] The cytologic method helps to distinguish heavy folds from infiltrative carcinoma. It is of aid in the differential diagnosis of the contracted distal stomach, an area that may be particularly difficult to interpret by X ray and gastroscopy.[88] It reveals a postoperative recurrence, but cannot be expected to detect the extraluminal growth of metastases. A hopeless prognosis of advanced gastric carcinoma may be altered by the finding of lymphoma cells.[86]

Cytology should be employed annually in all patients with pernicious anemia to find the precancerous polyps and carcinomas of the stomach that are known to occur with increased frequency in this disease.[24] The carcinomas complicating pernicious anemia may be of the slow-growing polypoid type. If detected in the asymptomatic phase, they should be curable.

Accuracy. The diagnostic accuracy of established cytologic centers is between 80 and 90 per cent in proved gastric cancer. False diagnoses of cancer should be kept to a minimum. In experienced hands, the false positive rate should probably be kept below 1 per cent.

Gastric cell collection and interpretation is difficult. Good results cannot be expected from the inexperienced or occasional observer. The trained personnel required compare with that necessary to operate a competent diagnostic radiology service. The valuable clinical results obtained in several centers[2, 50, 62, 89] would merit expansion of present facilities for gastric cytologic diagnosis.

Future research. For years, the stomach has been relatively inaccessible to dynamic studies of mucosal morphology.

Gastric X rays may be repeated with minimal discomfort to the patient. Nevertheless, the information afforded is relatively gross. The barium silhouette of the gastric folds is more likely to reflect muscular tone than fine mucosal morphology.[90]

Portions of the surface of the living stomach are brilliantly visualized by gastroscopy. Small precancerous polyps and shallow ulcers not visible on X ray may be revealed. It is not easy to follow a changing gastroscopic picture, because many patients will not tolerate repeated examinations. Furthermore, the gastroscopic interpretation of the visible mucosal surface does not necessarily correspond to the more objective information obtained by histologic examination of the full mucosal thickness.[24, 91]

Serial gastric suction biopsies may be taken with relatively little discomfort to the patient.[92] The histologic material obtained is excellent, but the site of biopsy is blind and, if the mucosa is atrophic, the danger of bleeding is not inconsiderable.

A simple cytologic procedure such as chymotrypsin lavage is harmless and well tolerated by the patient. The lavage fluid bathes most of the gastric mucosa and probably yields a representative sample. Repeated cytologic examinations reveal a dynamic picture of pathologic processes as they evolve. Exfoliated cells do not give the architectural information afforded by a tissue section. It is desirable to correlate the cells with the tissue obtained by blind suction biopsy, surgical excision, and necropsy.

Patients with pernicious anemia presumably have a hereditary gastric defect that manifests itself by deficient intrinsic factor production and by an increased tendency to develop gastric polyps and carcinomas.[93] This disease is particularly suited to studying the pathogenesis of gastric carcinoma.

Cytologic examinations of the stomach in pernicious anemia are indicated at annual or more frequent intervals.[24] Carcinoma of the stomach has been observed to develop during the course of such serial cytologic studies.[94]

In three cases of *treated* pernicious anemia with gastric cancer, a wide spectrum has been observed from the characteristic large cell to definite adenocarcinoma.[24, 69] These findings pose several questions: Are these large gastric columnar cells the progenitors of the carcinoma, or do they exist in areas separate from the carcinoma and merely represent the substrate in which polyps and cancer are more prone to develop? In this laboratory, the transitions from normal to large bland columnars has been observed in a single sheet of exfoliated cells. A similar transition from the large bland to the large active form has been noted. A further transition in contiguous cells from the large benign form to frank carcinoma has not been seen to date.

The theory that large active cells indicate polyp[24] merits testing in a larger, statistically significant series.

Gastric cytologic investigation of families with pernicious anemia may discover unsuspected subclinical cases. Similarly, a few patients with gastric polyps may have preanemic pernicious anemia. Anemia in this disease is but one manifestation of a systemic deficiency of vitamin B_{12}. The histologic appearance on gastric suction biopsy may lead one to suspect pernicious anemia in an early phase.[95, 96] An unsuspected preanemic case has been detected in this laboratory by its typical cytologic picture.[25] The diagnosis was confirmed by evidence of defective intrinsic factor production obtained by the Heinle[97] and Shilling[98] methods of quantitating alimentary absorption of vitamin $B_{12}Co^{60}$. A gastrectomy was performed on this patient because of a polyp. Histologic examination revealed a fascinating intermediate phase of fundal gastritis that had not progressed to complete atrophy.[25] This type of investigation may uncover the earliest changes of this precancerous gastric lesion.

The exfoliative cytology of the stomach in severe B_{12} and folic acid deficiency bears some morphologic resemblance to gastric cancer despite its therapeutic reversibility. Moreover, atypical epithelial hyperplasia has been described in human folic acid deficiency induced by its antagonists.[27] These observations suggest further lines of inquiry.

It has been shown that achlorhydria is a useful test in the selection of patients to be screened for gastric cancer.[99] Initial cytologic studies of this group have shown large gastric columnar cells to be a frequent occurrence.[23] Yearly cytologic examinations of achlorhydrics should reveal some asymptomatic, curable gastric cancers. It should also yield other interesting data regarding precancerous changes.

The cytology of gastric polyps in patients who do not have pernicious anemia should be studied. It would be important to ascertain how their cytologic picture compares with that of pernicious anemia.

Living epithelial cells exfoliated from the stomach may be studied by phase microscopy without introducing artifacts of fixation and staining. Another challenging approach might be the *in vivo* culture of these cells. The differentiation of the various cell types alluded to above might be followed. It might be possible to observe the metamorphosis of one type to another. Unfortunately, the prevention of dedifferentiation and the promotion of further differentiation in tissue culture is beset with technical difficulties.

Organs Draining into the Duodenum: Liver, Bile Ducts, Gall Bladder, Pancreas

The work of Lemon and Byrnes[100] demonstrated the diagnostic value of cytologic examination of the duodenal contents in selected cases. Although the accuracy does not approach that obtained in the stomach and colon, any aid is welcome in diagnosing the inaccessible malignancies of the liver, biliary tract, pancreas, and duodenum.

Cell collection methods. Collection of cells from the duodenum is the most difficult and most time-consuming of all the gastrointestinal techniques. At times, the intubation of the duodenum is anatomically impossible. The numerous techniques that have been proposed testify to the difficulty of passing the pylorus. In order to be certain that the tip of the tube is in the second portion of the duodenum, fluoroscopic control is essential. The duodenum is normally collapsed, making continuous suction impractical because the holes of the tube become plugged with mucosa. A special tube that incorporates an air vent will automatically unplug the tube by sucking in room air whenever it is transiently occluded.[101] In this laboratory, various drugs have been given to relax the sphincter of Oddi and to promote the flow of secretions from the liver, biliary tract, and pancreas. Clinically, the latter substances seem to make little difference in the diagnostic results. Nevertheless, the approach bears further investigation.

Morphologic cytology. Duodenal cells are obtained individually and in sheets. The exfoliation is especially profuse in the presence of inflammation. The nuclei of these low columnar cells may be pyknotic or may have evenly distributed chromatin in a ground-glass pattern. At times, the striated borders of intestinal absorptive cells are apparent. Contaminating cells from the upper gastrointestinal tract are also present. There are those who believe that normal cells from the liver, ductal system, gall bladder, and pancreas can be differentiated.[102] This has not been our experience. The exact source of malignant cells is not of clinical importance for their mere presence is usually an indication for surgery.

Indications. Precise indications for duodenal cytologic examination are difficult to define. If facilities are available, jaundiced patients should be examined early in the course of their disease before obstruction prevents the recovery of diagnostic cells. Ideally, patients should be examined in the preicteric phase. Unfortunately, these malignancies are rarely suspected clinically before jaundice supervenes.

Accuracy. Diagnosis of the few cases of malignancy involving the liver or biliary tract observed in this laboratory has been uniformly unsuccessful, although others report somewhat better results.[103] An accuracy of approximately 50 per cent has been found in our small series of carcinoma of the pancreas. The latter figure is comparable to those found in another larger series of duodenal aspirations.[103]

Future research. At the present time, duodenal drainage is the most tedious and least rewarding of the gastrointestinal cytologic procedures. X ray and pancreatic function tests have even less practical value in the diagnosis of these inaccessible malignancies. There is a pressing need for a simple, harmless method of visualizing the pancreas radiologically. Perhaps a radio-opaque dye can be found that will be selectively concentrated by the pancreas. A simple angiographic method is an alternative approach. Duodenal drainage and careful cytologic study will be most efficiently utilized in the future with the development of a practical method for detecting asymptomatic cases.

Colon

Distal colonic cancers, which constitute the majority of large bowel malignancy, may be diagnosed by rectal palpation or sigmoidoscopy. Tannic acid evacuation, air contrast, and improved spot-filming techniques make it possible to detect small lesions of the more proximal colon by X ray. Large bowel cancer is more susceptible to surgical cure than the malignancies of the upper gastrointestinal tract. This more favorable prognosis should prompt the clinician to use every means at his command to diagnose these lesions promptly and precisely. The cytologic method is technically difficult in the colon, but it has definite value in differentiating benign from malignant lesions and in detecting cancer in areas blind to other techniques. Recent work[2, 104, 105] has done much to renew interest in this area.

Cell collection methods. Cell studies are unnecessary in lesions accessible to sigmoidoscopic biopsy. The one exception is a diffuse papillary adenoma.[2, 3] A suction tip applied to the whole surface of this lesion may reveal malignant cells that might be missed by a chance biopsy. Cancers of the distal descending and sigmoid are easier to diagnose than those of the more proximal colon and cecum. Bader and Papanicolaou[105] were the first to diagnose a cecal carcinoma. Three others have been detected in our laboratory since that time, although several lesions of the cecum and ascending colon have been missed.

The colon is prepared by the administration of two ounces of castor oil the noon before examination and by the use of enemas until clear the night before and the morning of the test. The patient should have nothing by mouth from the noon before examination. Glycerin may be used as an enema tip lubricant. Oily substances must be avoided, since they prevent cell adherence to the slide.

Sigmoidoscopy and barium enema should be performed prior to the examination in an effort to localize the lesion. Sufficient time must elapse between the X-ray examination and the cytologic study to avoid contamination by barium.

If the lesion is just beyond the reach of the sigmoidoscope, lavage may be performed through the instrument. Ringer's solution is poured into the scope, which has been inserted as far as it will go safely. Gentle inflation with air pushes the fluid into the more proximal colon. When the air seal is broken, Ringer's solution wells back into the scope and is aspirated. Tiny specks of tissue should be picked out and smeared directly on slides. The remaining fluid is centrifuged, and the sediment is smeared.

The whole colon may be lavaged by an enema of a liter or more of Ringer's solution slowly administered with the patient in moderate head-down position. The patient should be turned from side to side and the abdomen massaged to promote distribution of the fluid throughout the colon. After 10 minutes, the return is collected by gravity drainage and massage. The remaining fluid may be evacuated by the patient. Gross tissue particles should be smeared directly. The rest of the solution may be centrifuged and the sediment smeared. With the aid of a flexible obturator, one may attempt to insert the tip of the tube to the level of the splenic flexure in order to facilitate collection of cells from more proximal lesions.

Morphologic cytology. Fecal contamination of the smears is discouragingly frequent despite careful preparatory cleansing of the colon. Diagnostic cells may be rare and poorly preserved. Scanning of colonic slides requires extraordinary patience.

There are three types of normal cells that originate in the colon: columnar, goblet, and squamous cells. The columnar cells are often degenerate, exhibiting nuclear pyknosis, cytoplasmic eosinophilia, and fragmentation. Goblet cells are rare, but may be present in large numbers in association with cancer.[105] Squamous epithelium originates in the anal skin.

Well-preserved benign columnar cells are more frequent in inflammatory conditions such as diverticulitis. A few leukocytes are found in all patients. As might be expected, they are more numerous when infection is present. Red cells are not particularly diagnostic. Aggregates of lymphocytes are rarely seen and their significance is obscure.[105]

Endamoeba histolytica is clearly delineated by the Papanicolaou stain. Several incidental diagnoses of amoebiasis have been made in this laboratory. It is probable that other parasites may be detected in this manner.

Cells exfoliated from colonic adenocarcinomas present the usual nuclear aberrations.[17, 18] Experience has shown that the cytologic abnormalities required for the diagnosis of colonic carcinoma may be less outspoken than those needed to diagnose cancer of the stomach.

The varying morphology of malignant colonic cells may offer some clue to the histology of the tumor from which they originate. Two varieties of malignant cells were seen in a patient with a small pedunculated polyp of the upper descending colon. The usual picture of adenocarcinoma predominated. Other cell groups (FIGURE 10) were interpreted as early pearl formation, indicating squamous carcinoma. The excised polyp was a mixture of squamous and adenocarcinoma. Frondlike cellular aggregates (FIGURE 11) have been found in large numbers in a patient who proved to have a papillary adenoma with carcinoma *in situ*. Signet-ring cells have been observed in certain "signet-ring" carcinomas. Unusual malignant cytology in the colon may indicate a rare metastatic lesion. Large vesicular malignant nuclei have been observed in a cystadenocarcinoma of the ovary that had invaded the large bowel.

Indications. Colonic cytology is of greatest help in areas where the conventional diagnostic aids are weakest. It cannot be performed routinely because of the time and effort required.

Polyps beyond the reach of the sigmoidoscope may be discovered by a care-

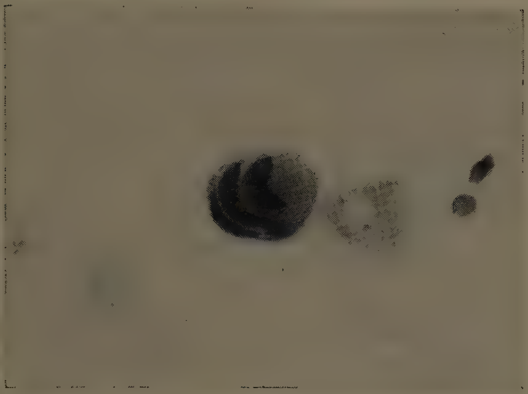


FIGURE 10. Colonic "pearl" (mixed squamous and adenocarcinoma). ×750.

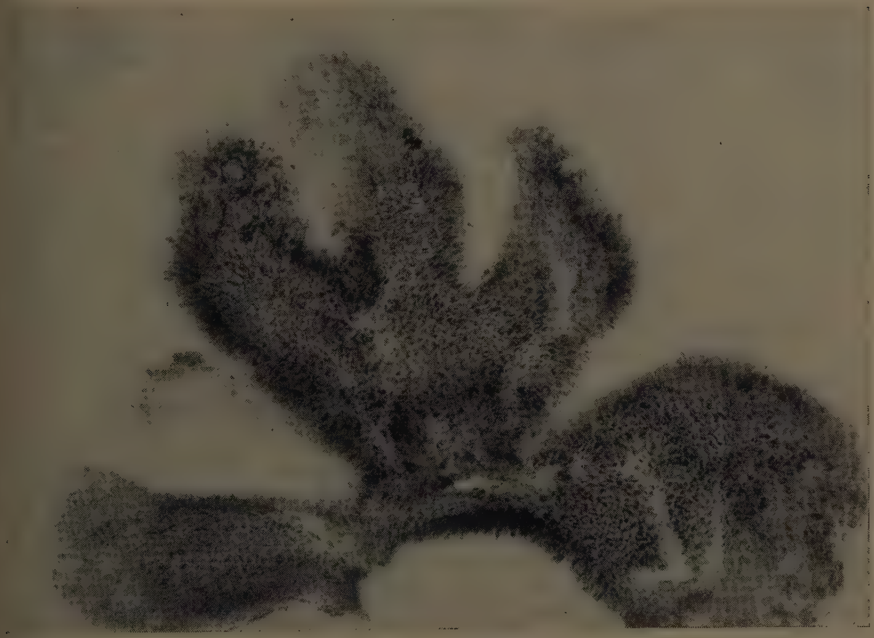


FIGURE 11. Colonic frond-papillary carcinoma.

fully performed barium enema. It is often impossible to determine if these are malignant by their gross radiologic appearance. The demonstration of malignant cells may prompt successful surgical excision in patients who would not ordinarily come to laparotomy.[2]

Carcinoma may be concealed within an area of diverticulitis. This is especially distressing, since the accompanying spasm of this disease usually prevents direct visualization and biopsy. Lavage *via* the sigmoidoscope is a practical approach that may make the diagnosis.[2]

In rare instances, malignant cells may be found despite a negative X ray and sigmoidoscopic examination. Two such cases have been observed in this laboratory.

After excising a colonic carcinoma, a constriction may develop at the site of the end-to-end anastamosis. Cytologic examinations help differentiate inflammatory narrowing from recurrent malignancy. Local recurrence merits a "second look," since the lesion may still be curable.

Carcinomatous changes in long-standing ulcerative colitis may develop. Unfortunately, these lesions are usually inoperable when diagnosed. Annual cytologic screening of these patients might prove profitable.

Accuracy. At present, cytologic accuracy leaves much to be desired in the cecum, ascending, and transverse colon. Lesions in these areas have been diagnosed.[2, 105] An increase in accuracy is to be anticipated with further refinements of cell-collection technique. Fortunately, 85 per cent of colonic cancers are located in the more accessible area, which extends from the splenic flexure to the anus.

Bader *et al.*[105] achieved the admirable accuracy of 95 per cent in proved cancer. The results in this laboratory were 82 per cent.[2] In compiling our statistics, polyps diagnosed as adenoma malignum presented a considerable problem. This term was used to describe polyps containing low-grade, non-invasive malignancy. For statistical purposes, these lesions were considered malignant. On the other hand, positive cytologic reports were reserved for the frankly malignant cells, which were only rarely present in adenoma malignum. In this laboratory, there are three possible cytologic reports: positive, negative, or technically unsatisfactory. It might be more realistic to add a suspicious category for the borderline lesions of the colon.

Future research. There has been little published in this area since the classic work of Bader and Papanicolaou. This is a challenging field, because the potential curability is high.

McGrew and Cole[106] smeared the proximal and distal ends of resected colonic malignancies. Despite a generous cuff of normal colonic mucosa, 42 per cent of the proximal and 65 per cent of the distal ends, when smeared, showed possibly viable malignant cells. This suggested that needle implants might account for their alarmingly high rate (10 per cent) of local recurrence, which was observed at the suture line. The placement of proximal and distal occlusive ligatures prior to manipulation remedied this situation.

Pomeranz and Garlock[107] took smears from the serosal surfaces of colonic tumors. Apparently, the minimal manipulation of smearing was capable of releasing apparently viable malignant cells. A colon cover with occlusive ligatures was suggested to minimize this source of peritoneal seeding.

By use of the cytologic technique, these workers revealed one possible source of postoperative recurrence. Occlusive covers might well be used in other types of cancer surgery. The demonstration of malignant cells at a distance from a grossly localized cancer should have the salutory effect of reminding surgeons to minimize operative manipulation.

Colonic cytology may be used for serial studies of the environment in which cancer develops. Some conditions suited to such studies are: familial polyposis, long-standing ulcerative colitis, and the postoperative colon after removal of carcinoma. Patients with adenomatous polyps discovered at routine sigmoidoscopy might be a fruitful group to follow. Interesting work in these areas is in process in several centers, and reports of progress should be forthcoming soon.

Summary

This is a review of the exfoliative cytology of the esophagus, stomach, duodenum, and colon. The varied methods of cell collection are evaluated. Normal and malignant cytology is described briefly. There is more detailed discussion of certain cells that have been reported recently. The clinical indications for cytologic examination are enumerated. Diagnostic accuracy in the various organs is analyzed. There is a discussion of current research, as well as speculation concerning future lines of investigation.

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THE CYTOLOGY OF ENDOMETRIAL CANCER

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Before the development of the cytologic method as a diagnostic approach to female genital cancer, the endometrium was relatively inaccessible. Vaginal and cervical smears, impressively accurate in detecting early cervical cancer, missed 40 to 50 per cent of endometrial malignancies.[1-6] With the introduction of the endometrial aspiration smear, the endometrial cavity was brought simply and painlessly into the realm of the accessible.

At the Gynecologic Cytology Laboratory of the University Hospital, from 1948 to 1953, 4350 endometrial smears were studied. In this group, 901 had tissue examinations. Included were 52 cases of cancer in which the cytologic diagnosis was accurate in 92 per cent.[7]

It has been demonstrated occasionally that a diagnosis of cancer made on the basis of the endometrial aspiration smear is accurate, even when prior or subsequent examination of curettings is reported negative. Six cases have been described wherein the endometrial smear indicated the presence of malignancy, in which the initial curettage or endometrial biopsy was negative or inconclusive. The cytodiagnosis in five cases was later confirmed by histologic examination although, in one instance, a second curettage was necessary.[8]

Since the earlier report of 52 malignancies, 49 additional cases of endometrial cancer have been studied at the Cytology Laboratory. In a total series of 101 cases, there were 15 errors in diagnosis. This makes it necessary to revise the accuracy of the method from 92 per cent down to 85 per cent as a result of further work.

Since the cytologic method has proved itself uniquely valuable in early cancer detection, it seemed of the utmost importance to investigate the causes of false diagnosis. It is the purpose of this paper to attempt to clarify the cytology of endometrial cancer by describing four confusing cell patterns that lead to errors in diagnosis;[2] to establish a more realistic basis for the role of endometrial smear diagnosis in cancer detection by recognizing its present limitations; and to reaffirm the value of the method.

At the University Hospital, indications for taking endometrial aspiration smears have been established as abnormal bleeding, during the menarche and postmenopausally. The method and materials used have been previously described.[9]

Although other staining procedures and fixatives are being tested, the standard Papanicolaou stains are used, since they insure sharp visualization of the nuclei, which is essential to accurate interpretation.[10, 11, 12, 13]

For simplicity, three criteria of malignancy have been established: hyperchromatism of the nuclei; irregularity of the chromatin network, with clumps of nucleochromatin distributed in different poles of the nuclei; and nuclear variation in size, shape, and staining reaction[14-18] (FIGURE 1).

No problem exists in evaluating endometrial cells when the smear pattern

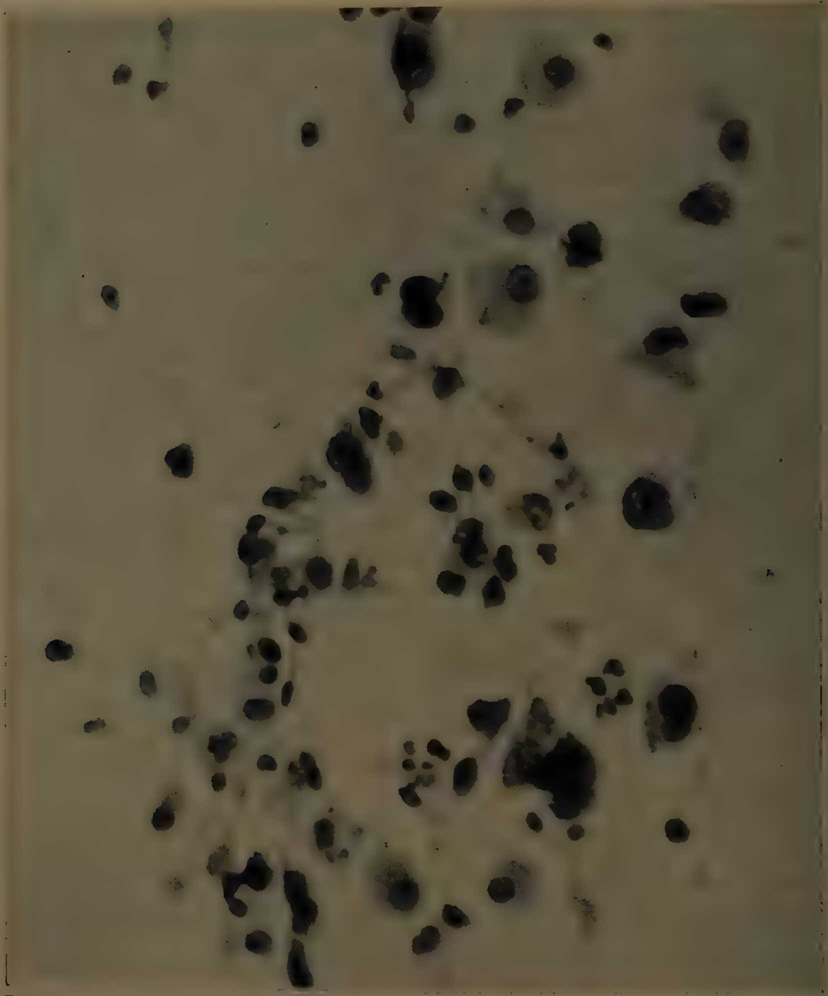

Figure 1. Adenocarcinoma of the endometrium showing nuclear hyperchromatism, irregularity of the chromatin network, and nuclear variation in size, shape and staining reaction.

satisfies the criteria just listed. It is when the cells suggest but do not conform unmistakably to the total picture of malignancy, that cytological confusion may result. For clarity, a need for classification of these confusing patterns became evident. An attempt has been made, therefore, on the basis of 9000 endometrial smears studied in a seven-year period, to classify recurrent confusing patterns into four main groups.

(*1*) *The first pattern is one in which hyperplastic cells are seen in close proximity to normal cells* (figure 2). As reported in an earlier study, endometrial hyperplasia has been accurately diagnosed by the aspiration smear in 75 per cent, confirmed by examination of curettings or of specimens secured at opera-

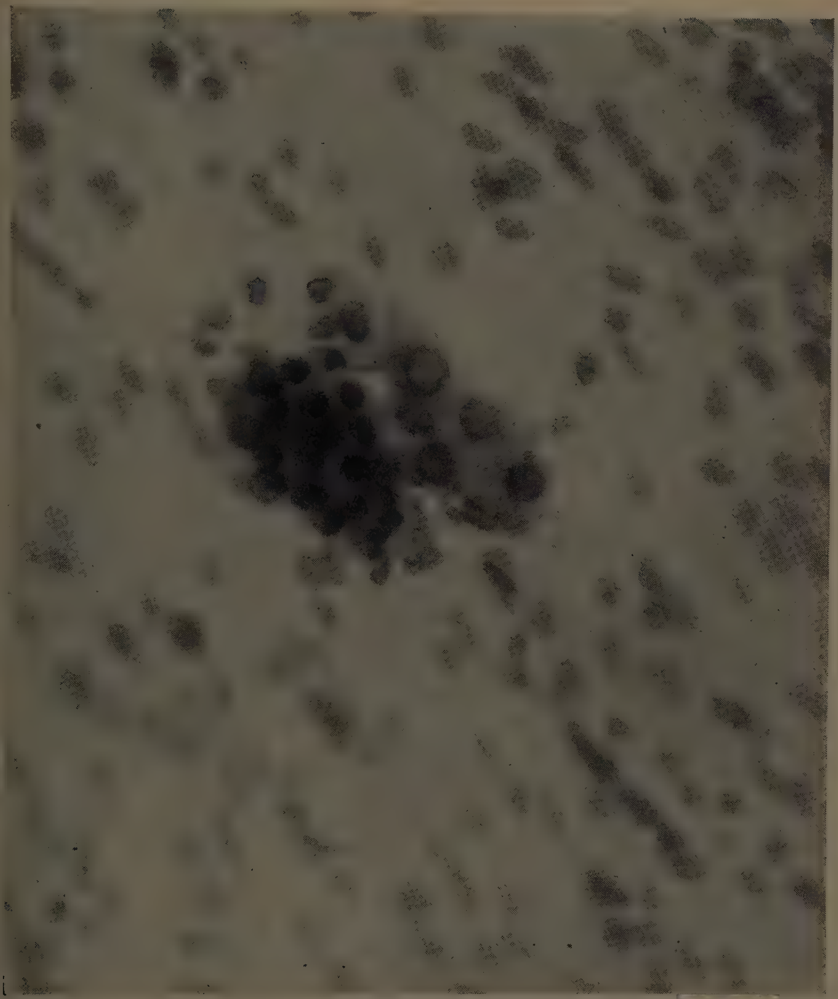

FIGURE 2. Pattern No. 1. Showing hyperplastic cells in close proximity to normal cells with apparent suspicious variation in nuclear size and shape.

tion.[9] Hyperplastic cells are seen as cells two to three times the size of normal cells, profuse numbers occur, the nuclei are large and vesicular, and there is a corresponding increase in the cytoplasm, but no disturbance of the nucleo-cytoplasmic ratio.

Hyperplastic cells present no problem in interpretation. When, however, they are seen adjacent to normal endometrial cells, which are smaller and have denser-staining nuclei, there is apparent suspicious variation in size and shape. Moreover, the presence of large numbers of cells is characteristic of the malignant pattern.

On the other hand, two important characteristics of malignancy are *not*

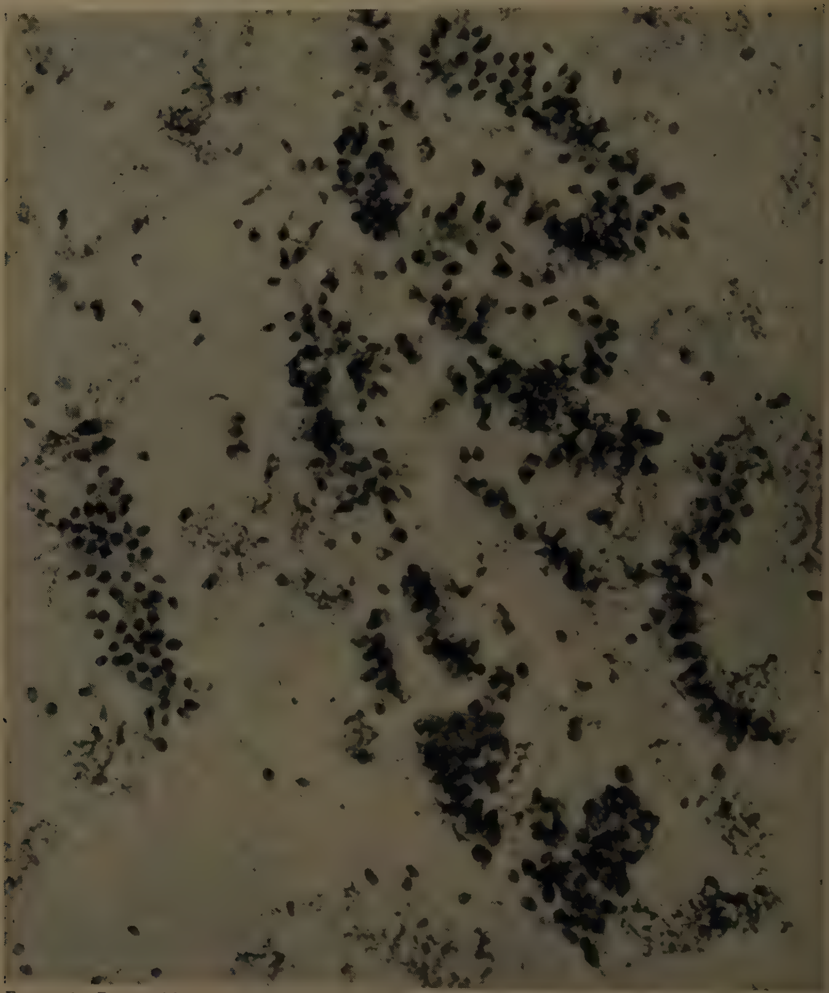

FIGURE 3. Pattern No. 2. Showing variations in endometrial cells as a result of uneven cyclic activity.

present: irregularity of the chromatin network, and hyperchromatism. The interpretation of hyperplastic cells together with normal cells on the same smear as malignant cells was responsible for seven false positives.

(*2*) *The second confusing pattern is caused by the fact that cyclic changes produce variations in endometrial cells* (FIGURES 3 and 4). These are variations in size and shape. The endometrium may show cyclic changes that do not conform temporally to current ovarian activity.[19, 20, 21] In normal smears, endometrial cells reflecting proliferative changes may be found in conjunction with cells characteristic of secretory activity. The former contain uniform nuclei with practically no vacuolization of the cytoplasm, whereas the latter show basal nuclei, slightly enlarged and indented due to marked swelling and

FIGURE 4. Pattern No. 2. Showing clusters of endometrial cells with apparent nuclear and cytoplasmic variations as a result of uneven cyclic activity.

vacuolization of the cytoplasm. The nuclei of some of the cells take a dense stain. Others present a pale, vesicular appearance. When uneven cyclic changes exist, many endometrial cells are degenerated, further complicating the over-all pattern of variability of the smear. This suggests malignancy.

Despite the report of one author that cyclic phases of the endometrium can be successfully determined by cytologic examination,[22] the foregoing observations do not bear this out.

The dilemma of the cytologist may be understood when uneven or normal cyclic changes are reflected in variation in the size and shape of normal endometrial cells and are associated with some variation in staining reaction and

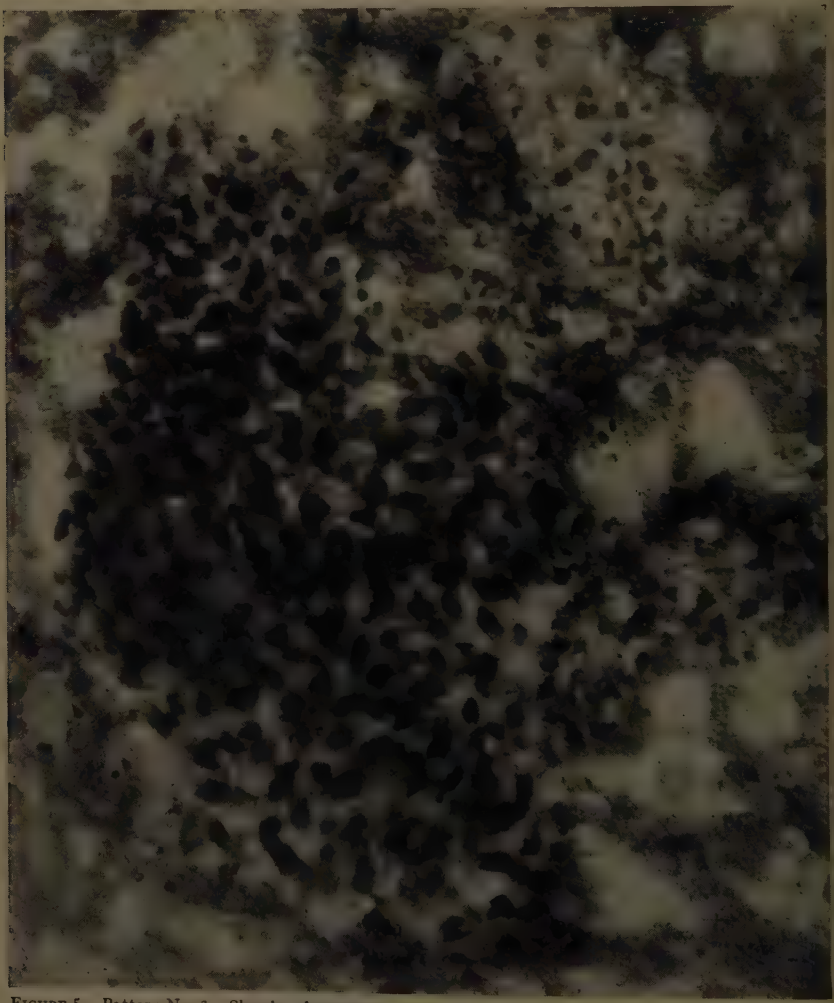

FIGURE 5. Pattern No. 3. Showing degenerated endometrial cells characterized by pyknosis, karyorrhexis and slight nuclear variation in size and shape which simulate malignancy.

with some degree of nucleochromatin irregularity. Considerable experience is required to identify such variability and to distinguish it from the picture seen in cancer. The pattern just described led to two false positives.

(*3*) *The third puzzling endometrial cell pattern involves degenerated cells* (FIGURE 5.) These are more frequently associated with postmenopausal women, particularly when some degree of cervical stenosis is present. The endometrial aspiration smear is a recommended procedure in postmenopausal bleeding. Smears from such cases may exhibit endometrial cells in various stages of degeneration, characterized by (1) pyknosis and intense staining of the nuclei; (2) karyorrhexis, or breaking up of the nucleus, which gives the impression

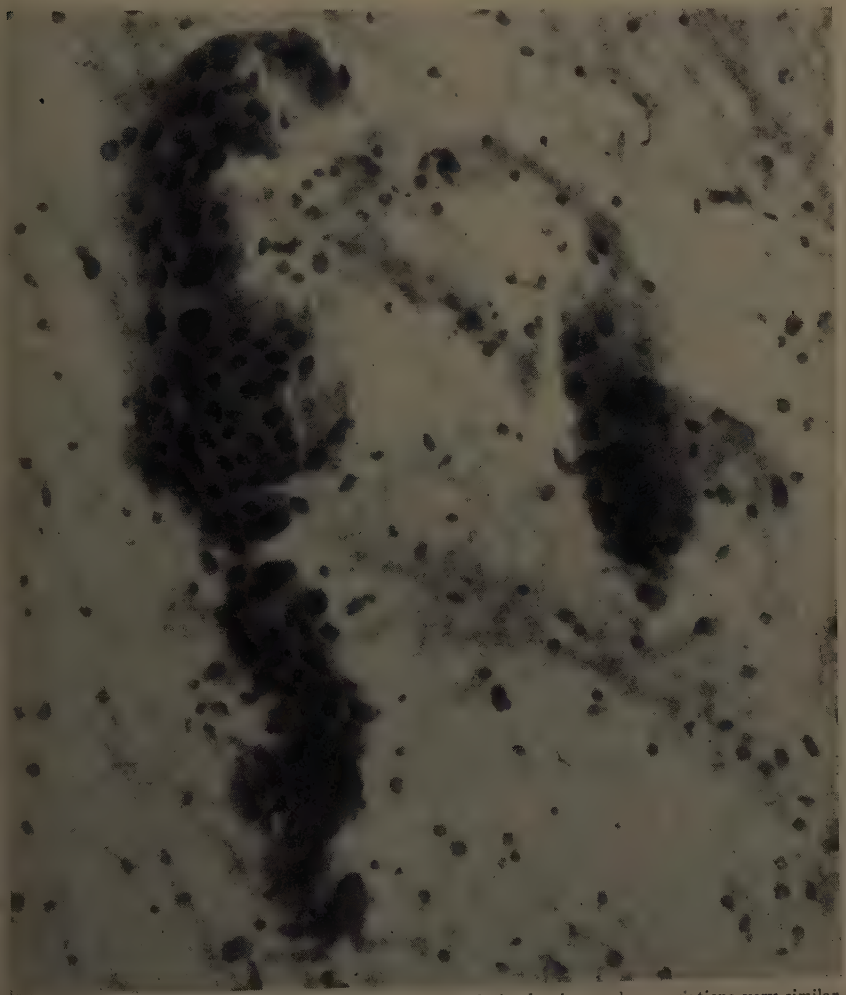

FIGURE 6. Pattern No. 4. Atypical endometrial hyperplasia showing nuclear variations very similar to those seen in adenocarcinoma.

of an unequal distribution of nucleochromatin; (3) slight variation in the size and shape of the nuclei.[23]

Normal atrophic endometrial cells are usually sparse on the smear. They occur in clusters in a mosaiclike pattern, the individual cells being somewhat smaller than normal. The nuclei may be round, elliptical, and pyknotic, and the cytoplasm is usually very scanty. The absence of two factors, hyperchromatism and irregularity of the chromatin network, will clarify the third equivocal pattern, which was responsible for three false positives.

(4) *The fourth confusing smear pattern is one of atypical endometrial hyperplasia* (FIGURES 6 and 7). This smear reveals profuse numbers of endometrial

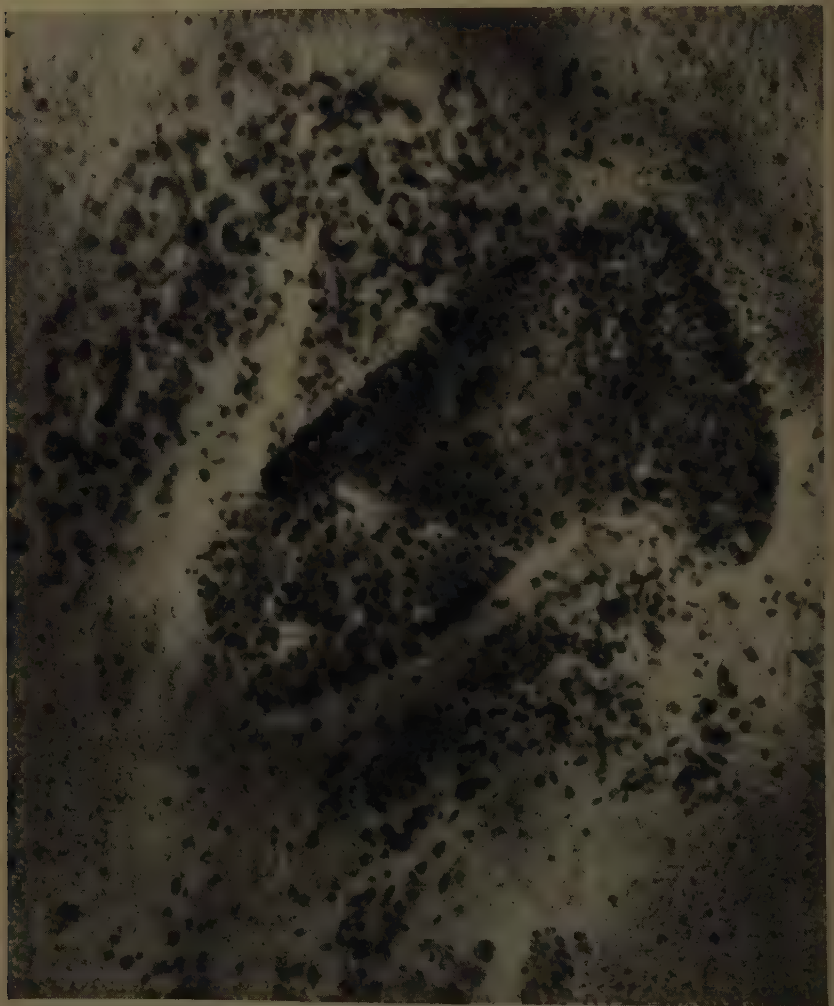

FIGURE 7. Pattern No. 4. Atypical endometrial hyperplasia. The abnormal cellular pattern is difficult to differentiate from that of malignancy.

cells, either singly or in clusters, with some degree of variation in size, shape, and staining reaction of the nuclei. A slight irregularity of the chromatin network is seen, and the total picture is strongly suggestive of malignancy. The morphology of the corresponding tissue section may be equally baffling to the pathologist.

The cells in this pattern are usually not very large. Hyperchromatism is nor marked, nor is there a notable irregularity of the chromatin network. In order to protect the patient whose smear presents this pattern, curettage and close observation with smears repeated at frequent intervals are recommended. These atypical changes may or may not be reversible. In lieu of further study, a report of atypical endometrial hyperplasia represents an "alert" to the cytologist and to the clinician, requiring their close cooperation. The cytologist tends to err on the side of caution. In the present study, this pattern accounted for three false negatives.

Although a diagnosis of malignancy is not made on the basis of one alteration in cell morphology, it should be noted that not all positive smear patterns satisfy all the criteria of malignancy. The diagnosis of cancer is a considered evaluation of the total picture. In the presence of marked inflammation, even cervical cells may show nuclear variations. Such evidence satisfies some of the criteria of malignancy, but not enough to warrant this diagnosis.

These limitations in the cytodiagnosis of endometrial cancer in no way diminish the validity of the method, which is a most valuable approach to cancer diagnosis. It is worth repeating, at this point, that a considerable amount of training and experience is required before a cytologist may be considered qualified.[24, 25] This necessary training and experience may also be regarded as a limitation.

It would seem that the degree of lack of accuracy is due, in large measure, to insufficient present knowledge of the significance of atypicalism in endometrial cells. Before the cytology of endometrial cancer can be brought into sharp focus, further investigations must be made, to differentiate the variability of normal cells, both single and in smear patterns, from the variability that identifies cancer. A diagnosis of cancer should never be made on the basis of the confusing patterns outlined in this presentation.

I look forward to the time when therapy may be instituted on a positive cytodiagnosis without confirmation by curettage, although that time has not yet come.

Conclusions

(1) The cytologic method in endometrial cancer diagnosis is at present 85 per cent accurate. When all criteria for malignancy are satisfied, an absolute cytodiagnosis will be substantiated by histological examination of the specimen.

(2) The limitations of the endometrial smear diagnosis have been indicated in the description of four confusing patterns that suggest but do not conform to the absolute picture of malignancy.

(3) It is hoped that this work will serve as an impetus to other investigators in clarifying the cytology of endometrial cancer.

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CYTOLOGIC EVALUATION OF BREAST SECRETIONS*

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Cancer of the female breast continues to be one of the leading causes of mortality in the United States. Public education, largely sponsored by the American Cancer Society, has had some success in alerting women to this disease by encouraging the practice of breast self-examination. During the past 10 years, much progress has been made in the treatment of carcinoma of the breast, but little, if any, has been made in diagnostic methods designed to detect mammary cancer in its early stages. Inspection and palpation still remain the chief methods of diagnosis. Transillumination is used mainly to distinguish cysts from other masses. Aspiration biopsy has also been utilized to determine the nature of an isolated mass in the breast or to confirm a clinical impression of cancer. The study of soft tissue X rays of the breast has been explored but appears to have a limited application. There is need for additional diagnostic weapons that can be used by the practicing physician. It is hoped that the accuracy of diagnosis of early mammary carcinoma will be increased with the aid of cytologic examination of breast secretion.

References in the literature on the study of breast secretion date back to the middle of the nineteenth century.[1] Since then, there have been many reports which, however, deal chiefly with the examination of colostrum. It is in more recent years that attention has been drawn to the diagnostic significance of breast secretion. Adair, in 1930,[2] pointed to the value of microscopic examination of breast secretion for determination of the presence of blood. Jackson and Severance[3] published a report in 1946 on the cytologic study of nipple secretions. Jackson continued this work and, with Todd and Gorsuch,[4] reported on the value of cytologic study of breast secretion for the detection of silent papilloma. Observations on the normal and malignant exfoliated cells found in breast secretion have been reported by Saphir[5, 6, 7] and by Papanicolaou.[8, 9]

This present report is based on the results of the examination of over 1600 women patients from New York Hospital, Memorial Center, and private physicians. Spontaneous nipple discharge was present in some of these patients. In the remaining patients, an attempt was made to obtain nipple secretion by careful palpation, massage, or by use of a hand breast pump. These maneuvers were performed gently to prevent trauma to the breast.

Approximately 900, or over 50 per cent of these 1600 patients, were found to be secretion-positive. The term "secretion-positive" is used to designate those

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* Aided by a grant from the American Cancer Society, New York, N. Y.

TABLE 1

ANALYSIS OF RESULTS OF EXAMINATION OF 656 ASYMPTOMATIC WOMEN

	Total No. of cases		Secretion obtained		Percentage	
Premenopausal						
Regular periods		273		73		26.8
1–14 days of cycle	144		35		24.3	
15–28 days of cycle	129		38		29.4	
Irregular periods		98		19		19.4
Total		371		92		24.8
Postmenopausal						
1–5 years		133		20		15.04
6–10 years		86		8		9.3
11–15 years		41		3		7.3
16 years and over		25		2		8.0
Total		285		33		11.6
Total of all cases		656		125		19.1

patients from whom secretion was obtained. The above percentage should not be interpreted as representing that to be expected in the general population, since symptomatic patients with spontaneous secretion were included in this study. If, however, the women examined are divided into symptomatic and asymptomatic groups, the asymptomatic group may be considered as more nearly representative of a sampling of the general population.

TABLE 1 shows an analysis of the results of the examination of 656 asymptomatic women seen at the Strang Clinic of Memorial Center. In this group of patients, the percentage of secretion-positive cases was 19.1 per cent. Interesting variations are noted when these asymptomatic patients are divided into premenopausal and postmenopausal groups. It is seen that, in the premenopausal group of 371 patients, secretion was obtained in 92, a percentage of 24.8, whereas in the postmenopausal group of 285 patients, only 33 or 11.6 per cent were found to have secretion. It has also been observed that, in the premenopausal women, the stage of the menstrual cycle appears to be a significant factor in obtaining nipple secretion. The greatest number of secretion-positive cases was found among women during the luteal phase of the cycle.

The cytology of abnormal breast conditions, including cancer, cannot be properly evaluated without an adequate knowledge of the cytology of the clinically normal breast. The two cell types most commonly present in secretion smears from normal breasts are the foam cells and the duct epithelial cells. Small numbers of histiocytes and leucocytes also may be seen. The foam cells exhibit considerable variation in size and shape. The larger ones have an abundant cytoplasm that has a finely vacuolated, foamy appearance (FIGURE 1). Their nuclei are usually eccentric. Occasionally, they may exhibit binucleation or multinucleation. Foam cells are seen in practically all smears from both normal and abnormal breasts, and are more numerous in some cases of chronic mastitis.

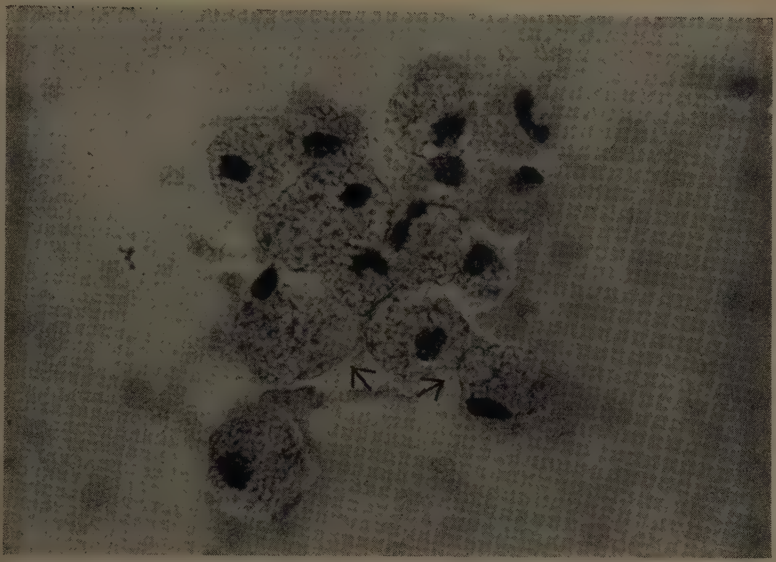

FIGURE 1. Foam cells. ×600. Note the erythrocytes in the substratum and within the cytoplasm of the cells.

Cells of this type have many characteristcs in common with histiocytes found in smears of secretions from other organs. Their phagocytic activity seems to be well established, and is illustrated in FIGURE 1 by the ingested erythrocytes in the cytoplasm. The vacuoles seen in the cytoplasm of foam cells are the result of dissolution of fat droplets by the alcohol-ether fixation. The presence of fat is demonstrated in FIGURE 2, taken from a smear stained with Oil Red O. The lipoid droplets in the cytoplasm can be seen clearly with the aid of this stain. Whether the presence of fat indicates secretory activity as claimed by Danesino[10] and others, or whether it is due to fatty degeneration or phagocytosis of fat, is still undetermined.

The typical picture of acute infection shows an overwhelming leucocytic response. In smears from cases of acute mastitis, the predominant cell is the polymorphonuclear leucocyte. Histiocytes also may be seen, as is usually observed in acute infections.

Desquamated duct epithelial cells and cell clusters are seen rather frequently in breast-secretion smears. The cluster seen in FIGURE 3 was found in the secretion from a normal breast. Cells similar in appearance may be found also in cases of proved intraductal papilloma, as shown in FIGURE 4. It is likely that these cells are derived from small papillary projections within the gland ducts and that, in some cases, they may actually represent a micropolypoid hyperplasia. Foote and Stewart[11] have reported that micropapillomatosis is not an infrequent occurrence. In a study of the nondiseased portion of both cancerous and noncancerous breasts, these authors observed that microscopic papillomatosis was present in greater frequency than the macroscopic papillomas.

FIGURES 5, 6, and 7 illustrate epithelial cell clusters from another patient

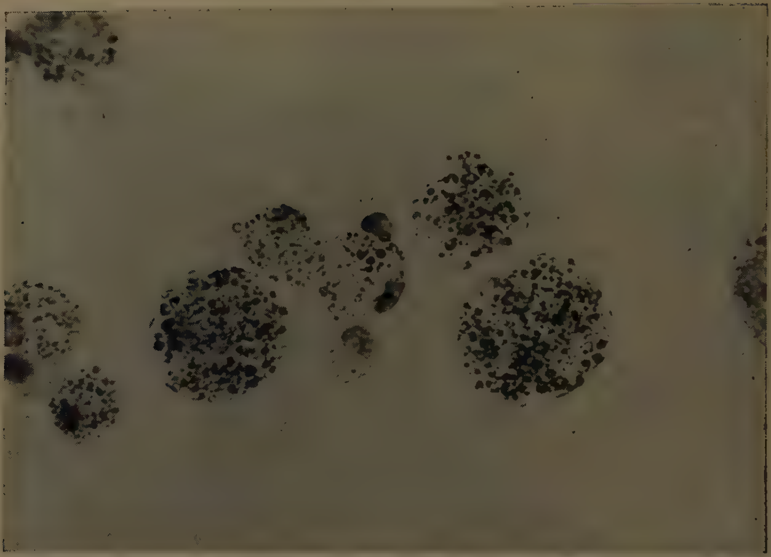

FIGURE 2. Foam cells stained with Oil Red O. ×600. The fat droplets appear black in the photograph.

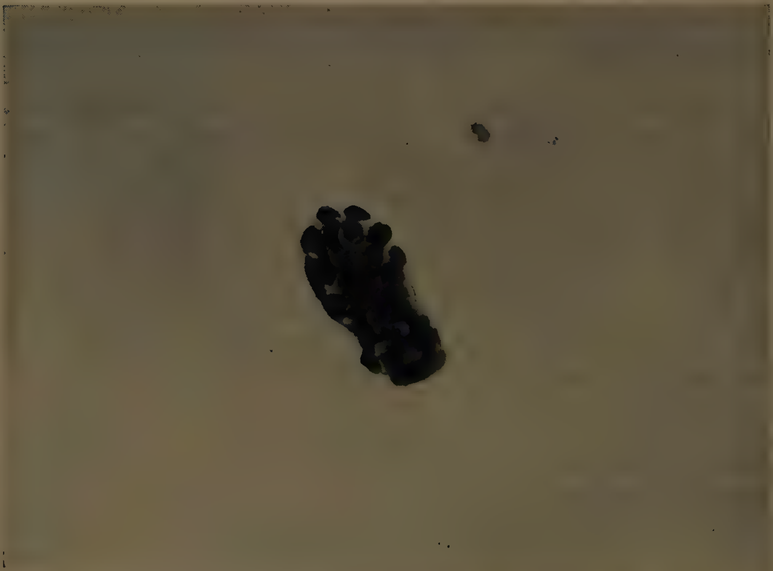

FIGURE 3. Cluster of duct epithelial cells from a clinically normal breast. ×600.

with proved papilloma of the breast. They demonstrate the variety in size and shape of cells exfoliated from this benign tumor. Occasionally, vacuolated cells are present. Erythrocytes are a common finding in smears from patients with papillomas. Histologic sections of this case are shown in FIGURES 8 and

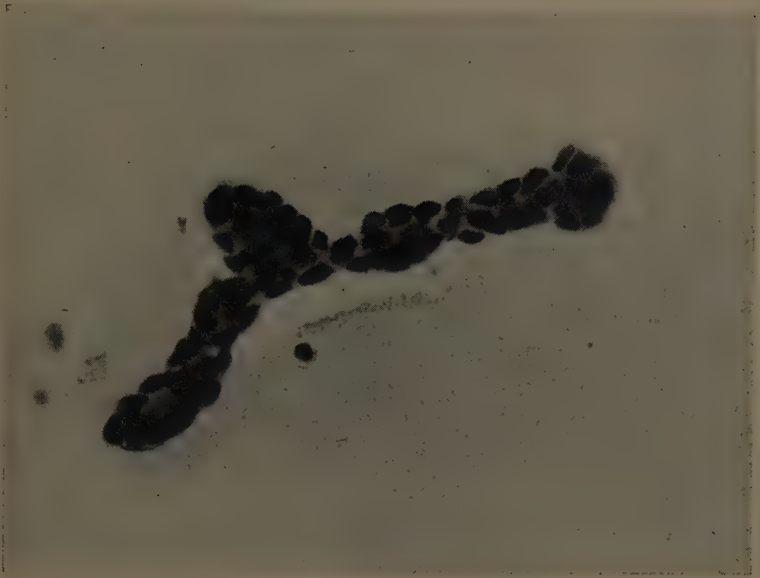

Figure 4. Cluster of duct epithelial cells from a case of intraductal papilloma. ×600.

9. The similarity of the cell-cluster pattern seen in the smears and the epithelial projections to be noted in the sections of intraductal papilloma is apparent.

Smears from cases of proved papilloma may sometimes contain cells exhibiting marked atypia. At times, the atypia is of such a character that it is difficult to determine whether or not malignant change is present. The pattern of the cell clusters may be no longer orderly, and there may be anisocytosis and anisokaryosis with some nuclear hyperchromasia, arousing suspicion of malignancy. Smears made from the breast secretion of a 52-year-old woman with the complaint of bleeding from the nipple of two weeks' duration were reported as showing evidence of papilloma with atypical features. An excision of the entire duct system was performed, and a number of separate papillomas found. The pathologic sections reflected the same border-line changes seen in the smears and were reported as "atypical papillomatosis; borderline malignancy."

Occasionally, cell clusters from a duct carcinoma and a papilloma may be seen in the same smear as shown in figure 10. Cell clusters characteristic of papilloma are seen intermixed with cells showing definite malignant features. This evidence still does not solve the problem of whether or not there is a direct relationship between papillomas and malignant lesions of the breast. Whether this case represents simply co-existence of these two lesions or a malignant change developing in a previously benign papilloma is an unanswered question. This case was diagnosed pathologically as duct carcinoma of the breast.

Figures 11 and 12 illustrate cells from a mammary duct carcinoma. The smears were taken from a patient who had no palpable mass in the breast. An excision of the duct system was performed on the basis of the positive smear

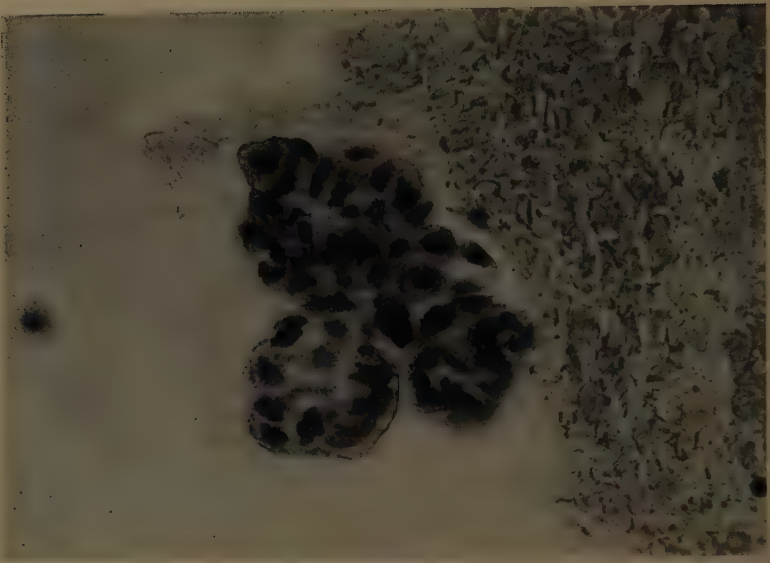

FIGURE 5.

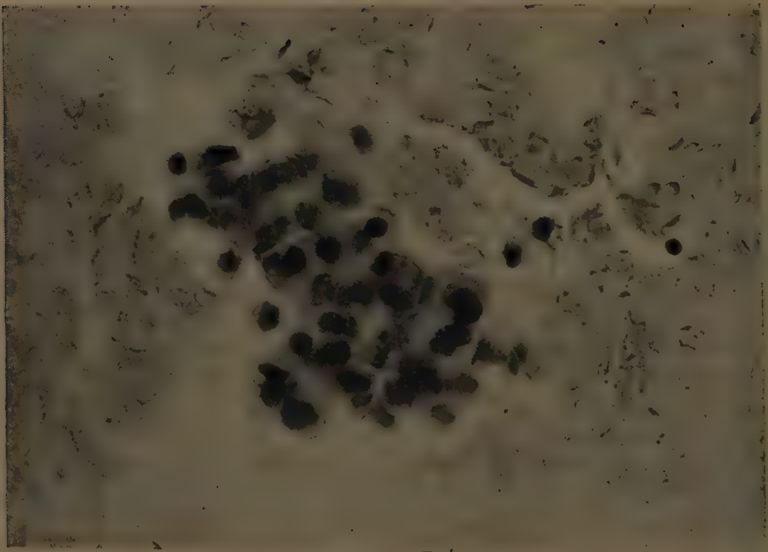

FIGURE 6.

FIGURES 5, 6, and 7. Epithelial cell clusters from a case of intraductal papilloma. ×600. Note the variations in nuclear and cell size and in cytoplasmic vacuolation.

report. Grossly, the specimen appeared to be negative but, on microscopic examination (FIGURES 13 and 14), the presence of an early carcinoma was established. The cytologic criteria for malignancy are seen here. The nuclei are enlarged and show irregularity in size and form, hyperchromasia, clumping of

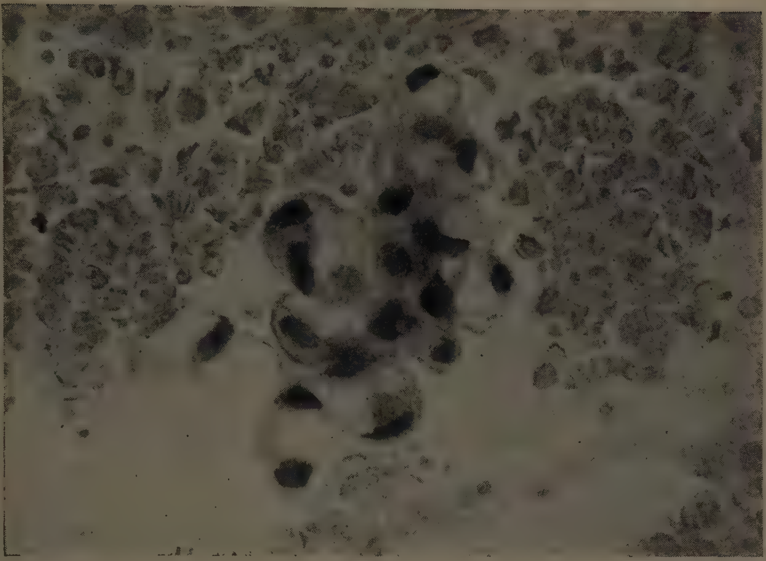

FIGURE 7.

the chromatin, and the presence of large, sometimes multiple nucleoli. This patient is alive and well five and one-half years following radical mastectomy.

Cell clusters from a similar case of early cancer of the breast are seen in FIGURE 15. The patient, who was completely asymptomatic, came to the

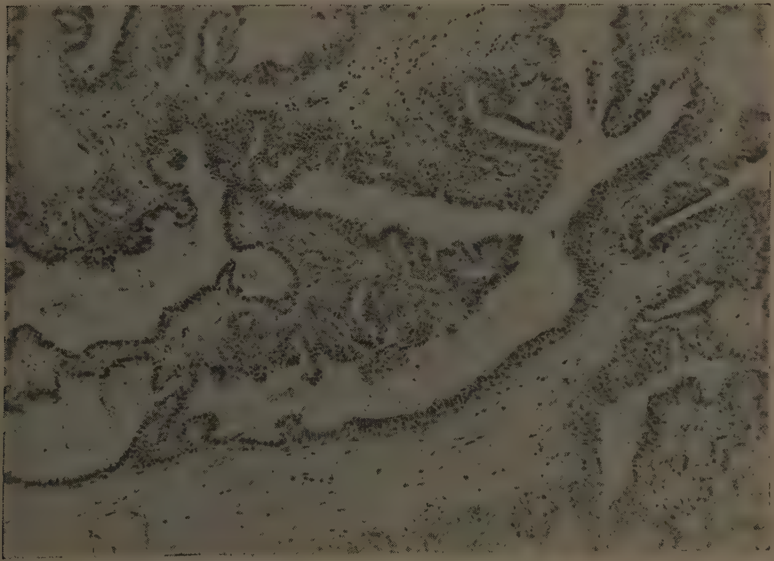

FIGURE 8. Low power photomicrograph of a tissue section of the papilloma. ×100. Same case as FIGURES 5, 6, and 7.

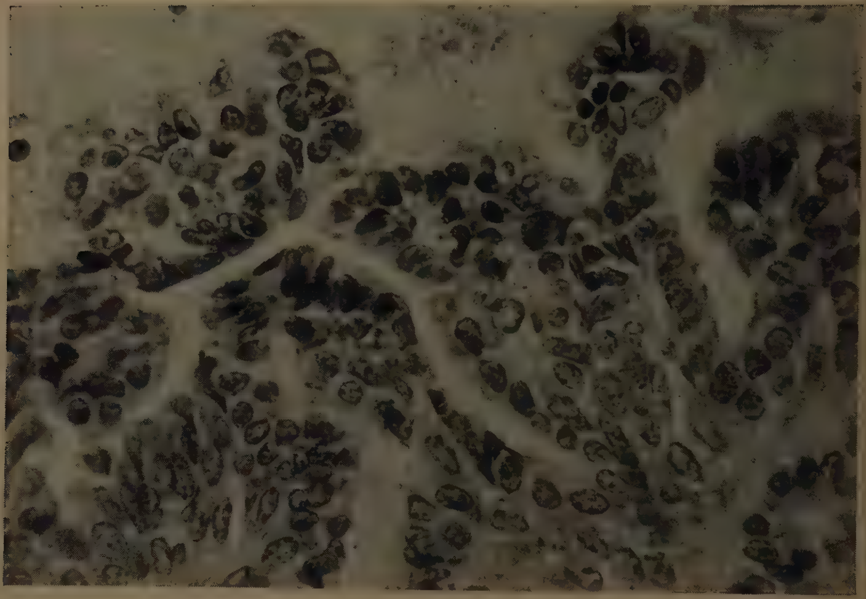

FIGURE 9. Magnification of an area from FIGURE 8. ×600.

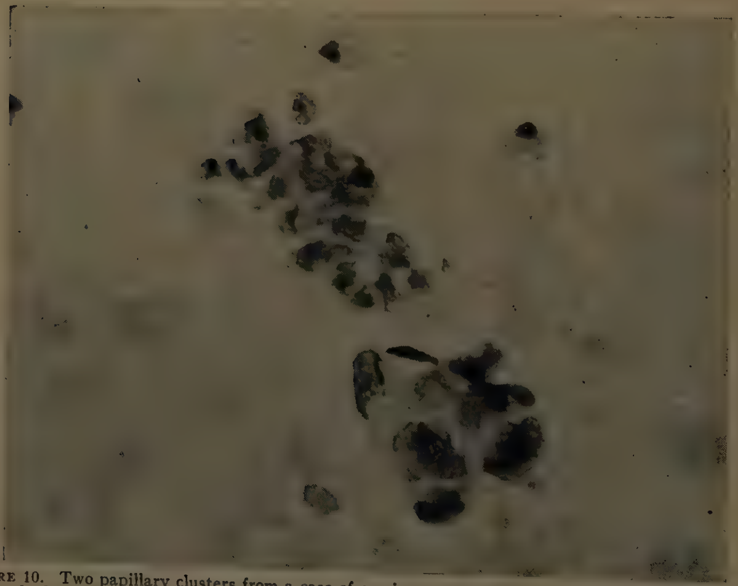

FIGURE 10. Two papillary clusters from a case of carcinoma. ×600. The upper cluster has a benign appearance; the lower is characteristic of carcinoma.

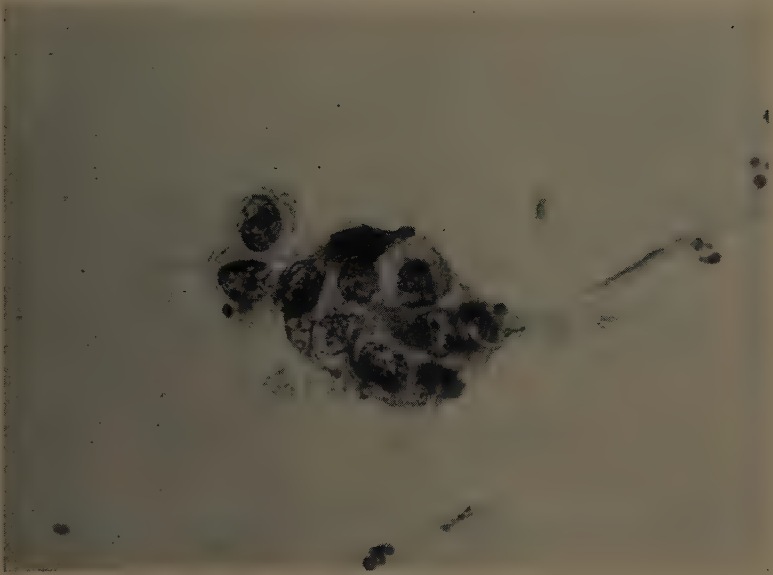

Figure 11.

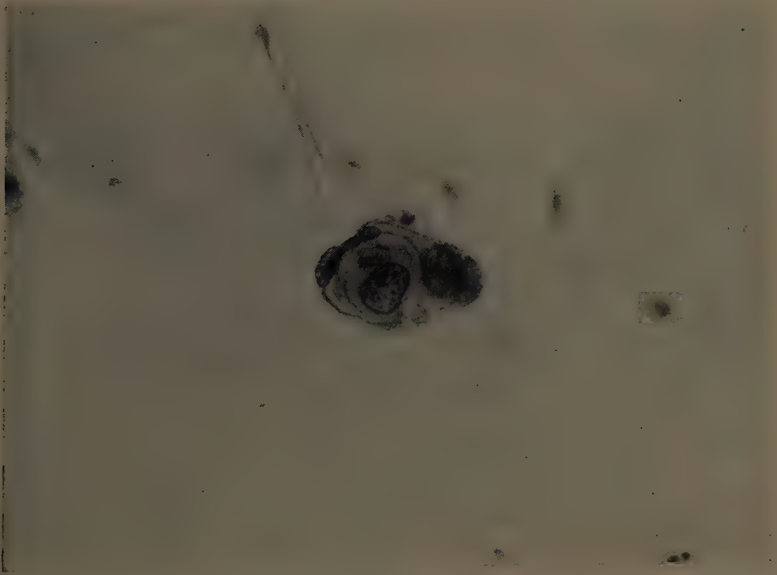

Figure 12.

Figures 11 and 12. Malignant cells from a case of early carcinoma of the breast. ×600.

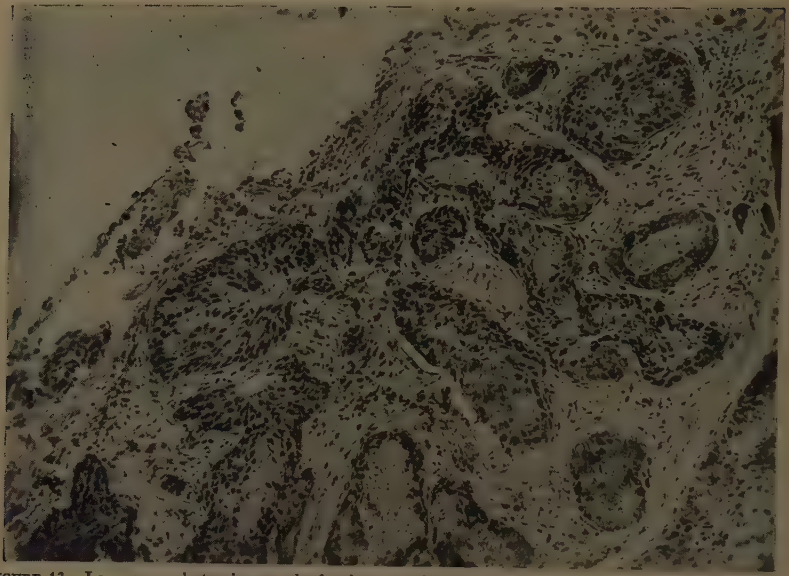

FIGURE 13. Low power photomicrograph of a tissue section of the carcinoma. ×100. Same case as FIGURES 11 and 12.

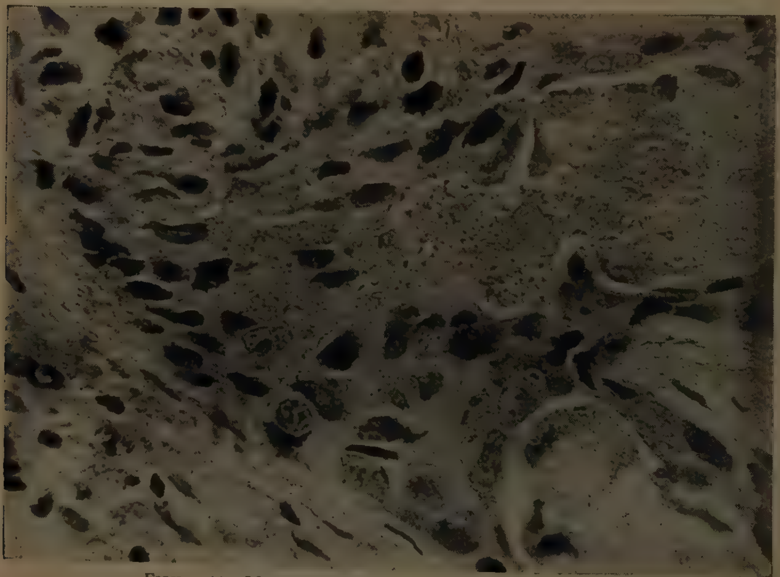

FIGURE 14. Magnification of an area from FIGURE 13. ×600.

Strang Prevention Clinic of Memorial Hospital for a checkup immediately after her mother was found to have carcinoma of the breast. During the examination of the breast, a small amount of secretion was seen exuding from the nipple. Smears were made from this secretion. Except for a minimal thicken-

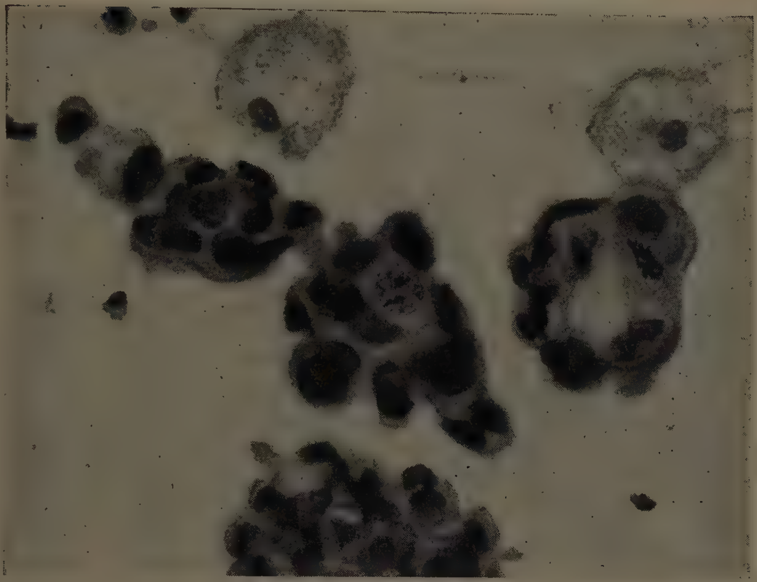

FIGURE 15. Malignant cells from an early carcinoma of the breast. ×600. Note the mitotic figure.

ing under the areola in the axis of 12 to 1 o'clock, the breast examination was completely negative. The smears were examined and reported as positive. The thickened tissue under the areola was excised, and frozen section was reported as "low grade carcinoma." The pathologic diagnosis of the mastectomy specimen was "comedo carcinoma of the breast" (FIGURES 16 and 17). This patient is free of disease more than two and one half years after surgery.

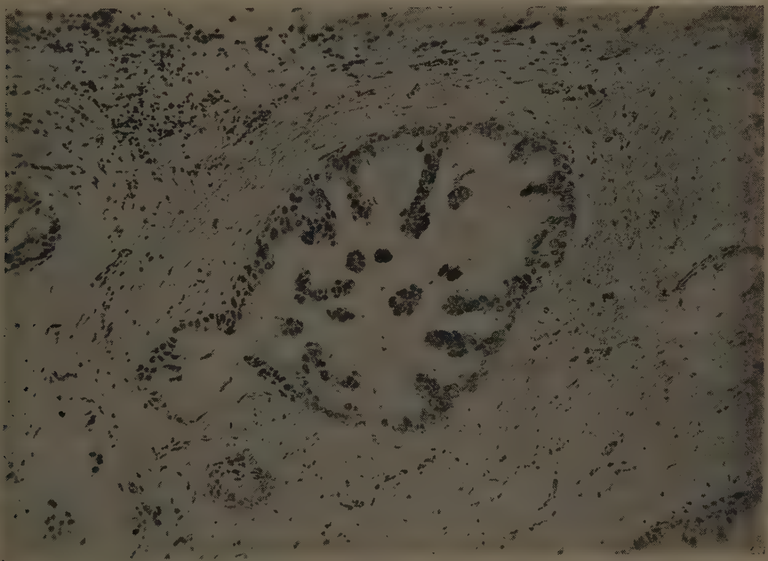

FIGURE 16. Low power photomicrograph of a tissue section from the case illustrated in FIGURE 15. ×100.

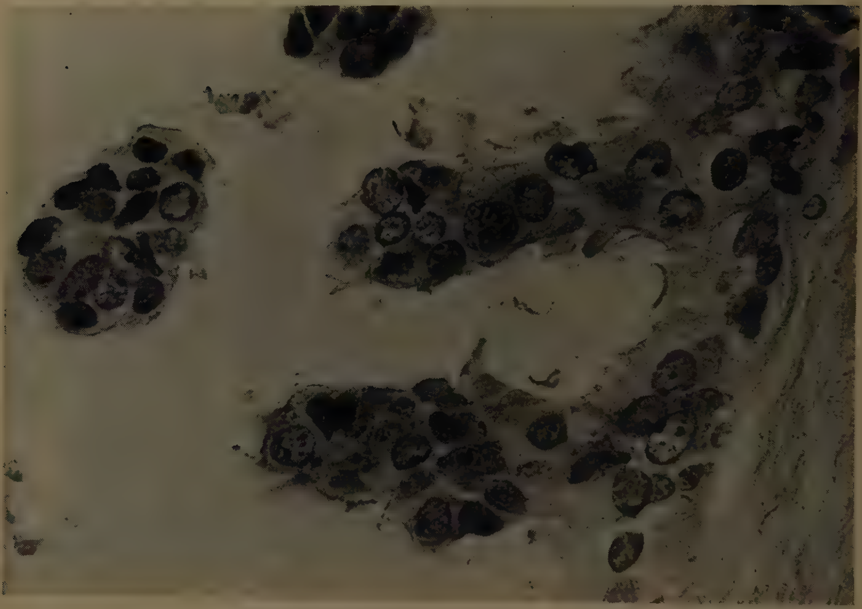

FIGURE 17. Magnification of an area of the section seen in FIGURE 16. ×600.

The goal of the cytologic method is to detect carcinoma in its early stage, when the prognosis for the patient is favorable. It has been observed that exfoliation in early lesions developing within the ducts is usually prolific. Two of the cases of early malignant tumors mentioned above were diagnosed primarily by the smear method in the absence of a palpable mass in the breast. Several single case reports on the detection of early carcinoma of the breast by the cytologic method are now on record.[12-16]

The clinical value of cytologic smears of breast secretion may be considered to be well established. The general criteria of malignancy as described for other organs appear to apply also to the breast.[8] The normal cytology of the breast is relatively simple in comparison with that of other organs. Normal cells exfoliated from the duct epithelium of nonpregnant women are usually small and show little variation in size and form. The absence of marked nuclear variability increases the significance of nuclear changes in this type of smear and reduces the possibility of false positive evaluations.

In this series, there have been no false positive reports. Our statistics, however, have shown a relatively high percentage (approximately 25 per cent) of false negative reports. In several of the cases missed by cytologic examination, the smears were very scanty or acellular. A factor affecting the recovery of malignant cells in the breast secretion is the location of the tumor, since malignant cells exfoliated from peripheral lesions may not reach the larger ducts. Errors may occur in the interpretation of the smears, as is true in other applications. These are most frequently referable to the difficulty of determining whether exfoliated papillary clusters with atypical morphology have been derived from a benign papilloma with atypical features or from an early duct carcinoma.

The cytologic method as a screening technique is still handicapped by the low percentage of secretion-positive cases, particularly in the postmenopausal group. However, in view of the fact that it is possible in some cases to diagnose cancer of the breast in its preclinical stage, the cytologic examination of breast secretion from all patients with nipple discharge appears to be mandatory.

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THE EXFOLIATIVE CYTOLOGY OF THE MAMMARY GLAND DURING PREGNANCY AND LACTATION*

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The present study was a natural outgrowth of a larger program of investigation of the exfoliative cytology of the normal and diseased human mammary gland (Papanicolaou, 1954). The purpose was: first, to describe the exfoliated cell types normal to the mammary secretion during pregnancy and lactation; and, second, to evaluate the possibility of utilizing the spontaneous secretion during these periods in screening for unsuspected carcinomas of the breast.

The study of smears of colostrum during pregnancy and after parturition is not new. As early as 1838, Donné made microscopic examinations of unfixed human colostrum and noted the presence of "granular bodies." To these granular bodies, Henle (1841) later gave the name colostrum bodies, a term that has been retained in most of the literature on this subject.

The majority of the subsequent papers have dealt with the origin and nature of the so-called colostrum bodies. Their origin has been described as mesenchymal (Bizzozero and Vassale, 1887), leucocytic (Czerny, 1890), histiocytic (Gruber, 1924), and epithelial (Gregoire, 1930 and Engel, 1953). Wallich and Levaditi (1905) and Varrier-Jones (1924) presumed that some colostrum bodies were derived from the epithelium and others from the mesenchyme. Still other authors (*e.g.*, Forsell, 1939) have felt that the colostrum bodies of the early investigators were not cells but merely agglomerations of fat globules.

A smaller number of papers have described the different cellular elements present during various stages of pregnancy and lactation. Lourié, in 1900, reported observations on the cytology of postpartum breast-secretion smears taken before and after the initiation of nursing. Five years later, Wallich and Levaditi described the cytologic differences in human milk during full lactation, slackening, and weaning of the infant. The most extensive material to date is that of Xhenseval (1949), who studied smears of mammary secretion from women in various stages of pregnancy from the first month to term, after parturition, following weaning, after abortion, and from seven women who were not pregnant.

The above reports deal chiefly with the relative number and structural variations of the single cells to be seen in the smears. With the exception of the work done by Xhenseval, there has been little attempt to compare the exfoliative cytology of the mammary gland during pregnancy and lactation with that of the nonpregnant state. As for the diagnostic use of breast-secretion smears in these stages, Xhenseval stressed their value in determining fetal deaths *in utero* and in detecting concealed abortions over a period of six weeks. No mention is made of their use in the detection of diseases of the breast.

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* Aided by a grant from the American Cancer Society, New York, N. Y.

Material and Methods

The present paper is based on the study of mammary secretion from 20 women by means of smears prepared at the time of their regular antepartum clinic examinations and during the one-week hospitalization following parturition. Postpartum smears from an additional 39 women were also studied. The authors wish to express their appreciation to Doctor R. Gordon Douglas, Obstetrician-and-Gynecologist-in-Chief, New York Hospital, for permission to obtain this material, and to Doctor Robert Ayerst for the physical examinations and the preparation of the smears.

The women selected for this study ranged from 17 to 43 years of age, with a mean age of 27.2 and the mode falling at 25. The majority were in their first or second pregnancy, with a smaller number in the third, fourth, sixth, and eighth. All women studied had clinically normal breasts and, in all cases, normal living infants were delivered, either spontaneously or by Caesarian section.

During pregnancy, secretion is most easily expressed in the latter half. Therefore, this period is better represented in our present series than is the first half. Of the six women examined during the first trimester of pregnancy, only two had secretion. Breast secretion was obtained by means of gentle palpation or by mild suction with a hand-breast pump. The drop of secretion appearing on the nipple was removed by touching to it a clean slide thinly coated with Mayer's egg albumen. The smears were routinely fixed in ether alcohol and stained by the Papanicolaou procedures. A few smears were fixed in 10 per cent neutral formalin or formalin vapor, and stained with Sudan IV or Oil Red O for the demonstration of fat.

Observations

In the present paper, we shall not attempt to correlate the various cytologic patterns with the specific stages of pregnancy. Instead, we shall confine our report to the more important findings as they relate to our understanding of the exfoliative cytology of the resting human mammary gland.

The breast-secretion smears during pregnancy and lactation are, in general, more cellular than those from clinically normal breasts of nonpregnant women. The appearance of the cells and the particular cell type predominating in relation to the cell population of the smear as a whole vary considerably, not only with the stage of pregnancy but also between individuals in the same stage and, sometimes, between smears from the right and left breasts of one woman. The cellular elements observed are essentially the same as those found in smears from resting glands; that is, the foam cells, leucocytes, histiocytes, and single cells and cell clusters desquamated from the gland epithelium (Papanicolaou *et al.*, 1955). They exhibit, however, definite alterations in structure and in frequency of occurrence.

Foam cells. The foam cells are common constituents of the breast-secretion smears in all stages studied. They undoubtedly represent what have been called the colostrum bodies by, at least, the more recent authors. The foam cells are not peculiar to pregnancy and lactation, however, having been described in all stages from the witch's milk of infants (Danesino, 1952, and others) to nipple discharge in postmenopausal women. For this reason, cells of this type will be referred to in this paper as foam cells rather than colostrum bodies.

In smears of all stages under study, some of the foam cells have the typical appearance of those found in smears from the resting gland. Frequently, however, they show structural modifications that are characteristic of the antepartum and early postpartum states. The changes noted are nuclear enlargement, increased frequency of binucleated and multinucleated forms, some cells containing as many as 35 to 40 nuclei, and heightened cytoplasmic vacuolation (FIGURE 1). In line with the greater cellularity of the smears, the foam cells are usually much more numerous during pregnancy and lactation than in smears from normal breasts in the resting state. They constitute the predominant cell type throughout the greater part of gestation in most cases.

Leucocytes. Polymorphonuclear leucocytes are the most frequently noted of the blood-cell elements. They are generally scattered throughout the smear (FIGURE 2) but, occasionally, they form large aggregates (FIGURE 3). Immediately prior to and just after parturition, the leucocytes are the predominant cell type in the smears. At this time, their appearance frequently becomes altered by the phagocytosis of fat droplets. The body of the cell is distended by a single vacuole, and the polymorphic nucleus is attenuated thinly over the surface of the vacuole. The contents of the vacuoles in the leucocytes, as well as in the foam cells, are stainable with Sudan IV and Oil Red O. Although the presence of leucocytes in large numbers is considered atypical in

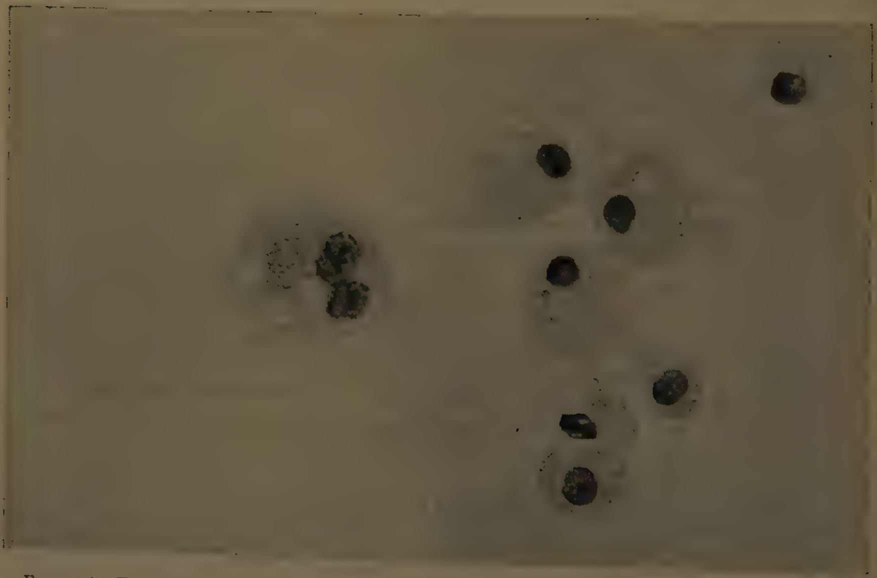


FIGURE 1. Foam cells. ×600. Note the variation in nuclear size, binucleation, and a few large vacuoles in the otherwise foamy cytoplasm.

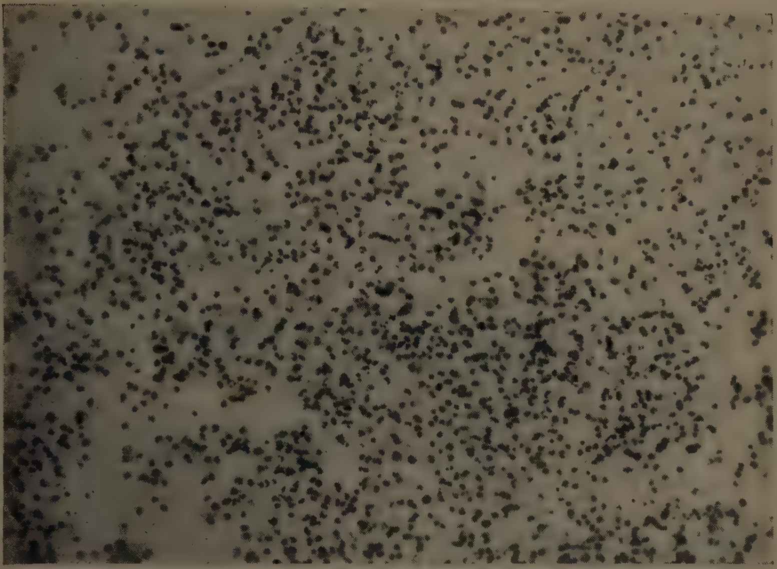


Figure 2. Low power view of a portion of a smear containing numerous leucocytes. ×160.

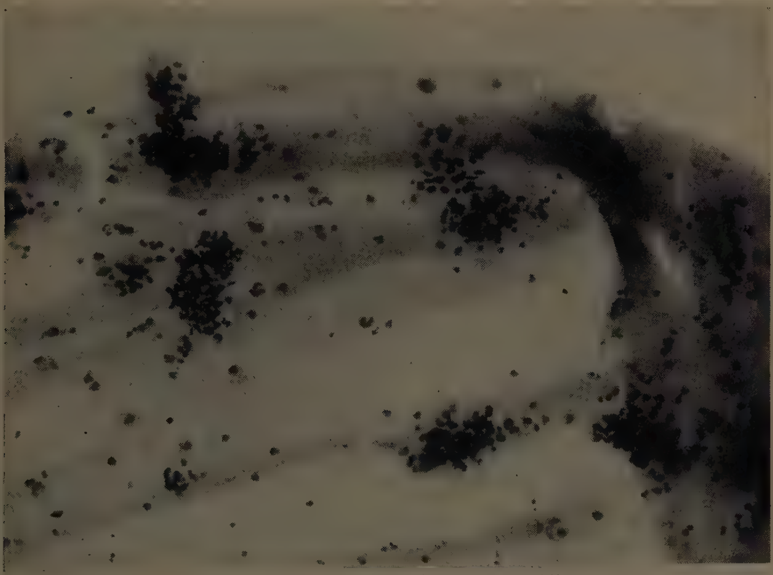


Figure 3. Aggregates of leucocytes. ×160.

smears from the resting mammary gland, it appears to be a normal occurrence in late pregnancy and following delivery.

Histiocytes. Histiocytes appear with less regularity and are not characteristic of any one period. Occasionally, they are present in large numbers and form a conspicuous part of the smear. More frequently, they are intermingled with the leucocytes and are scarcely noticeable except under high power inspection. When present, they may have a typical bean-shaped nucleus and a varying quantity of cytoplasm (FIGURE 4).

Epithelial cell clusters. An unexpected finding in the present investigation was the large number and the character of the cell clusters exfoliated from the gland epithelium during pregnancy and lactation. In so far as we have been able to ascertain, these clusters have have not been described in previous papers on prepartum and postpartum smears. A point of particular interest is that, in configuration, some are strikingly similar to fronds exfoliated from benign intraductal papillomata. For the sake of description, the clusters observed may be classified into five types, although there are, of course, transitional forms. These cluster types appear to represent stages of differentiation of the epithelial cells.

Cluster type A (FIGURE 5) consists of small, rather undifferentiated cells in a compact arrangement. The nuclei are small, deeply staining, though not pyknotic, and are surrounded by very little cytoplasm. This cluster type appears to be identical with that occasionally seen in smears from clinically normal breasts of nonpregnant women.

Cluster type B (FIGURE 6) also shows a compact arrangement of the cells. The nuclei are less dense, however, and are approximately twice the size of those in type A. The larger size of the cluster in FIGURE 6 as compared with the type A cluster in FIGURE 5 is due to the increase in nuclear and cell size, as the cell number is approximately the same. The nuclear-cytoplasmic ratio is about the same, with a relatively small amount of cytoplasm per cell in both

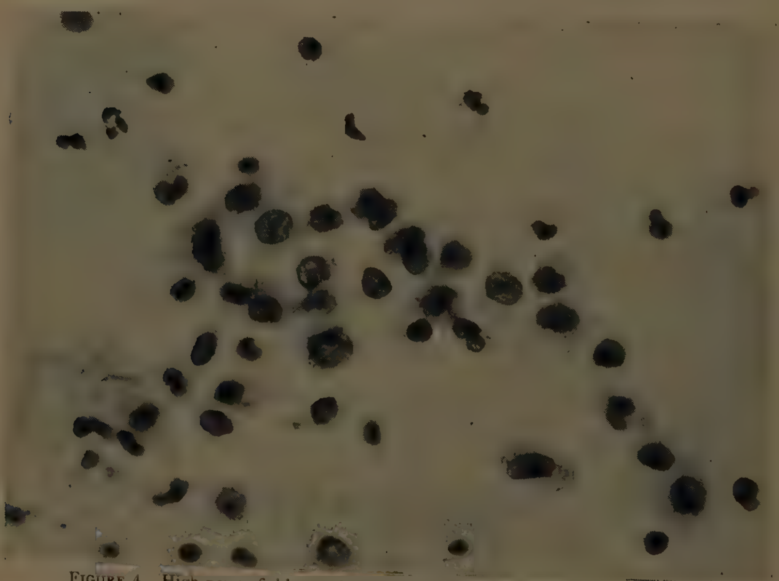


FIGURE 4. High-power field containing histiocytes and a few leucocytes. ×600.

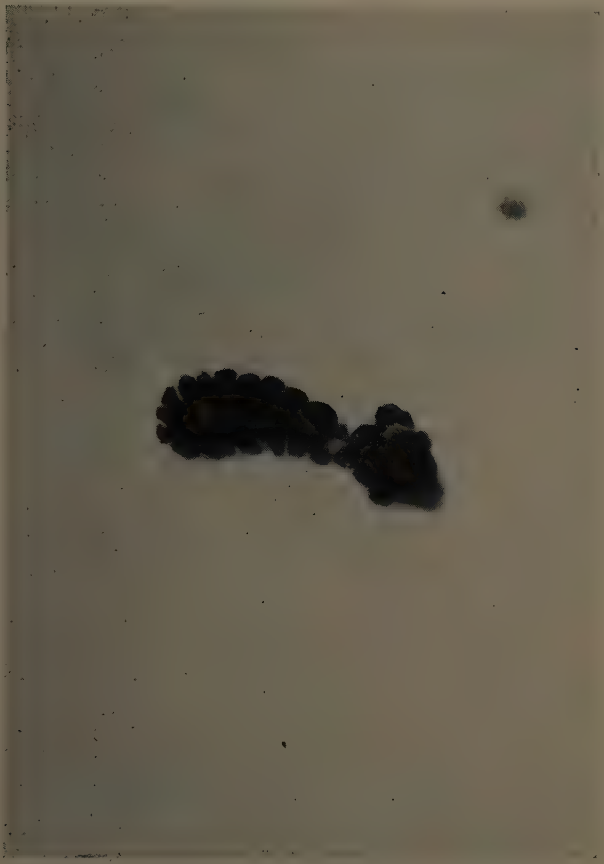


FIGURE 5. Duct epithelial cells. Cluster type A. ×600.

cluster types. Occasionally, a few cells in type B clusters contain single vacuoles.

The clusters of type C (FIGURE 7) differ from those of type B chiefly in the increased amount of cytoplasm per cell, since the nuclear size is approximately the same. Binucleation is not uncommon, and cells with vacuoles may be observed. In this type, the nuclei tend to be eccentrically located.

Type D clusters (FIGURE 8) exhibit a still greater increase in the amount of cytoplasm per cell with a decrease in the nuclear-cytoplasmic ratio. The cytoplasm is generally foamy, with fine vacuoles. Typically, there is a heavy ectoplasmic rim that is more noticeable in the peripheral cells if the cluster is compact. Apparently, the nuclear size is not increased over that in types B and C, but binucleation and multinucleation are common, with from one to six nuclei noted per cell. The nuclei in this cluster type tend to be pyknotic, although some fresh, well-preserved forms have been observed.

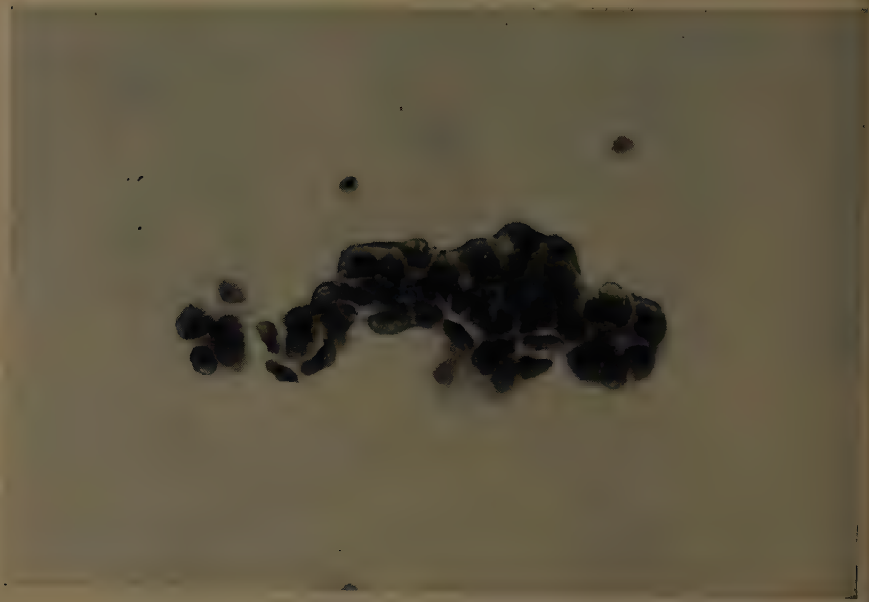


FIGURE 6. Duct epithelial cells. Cluster type B. ×600.

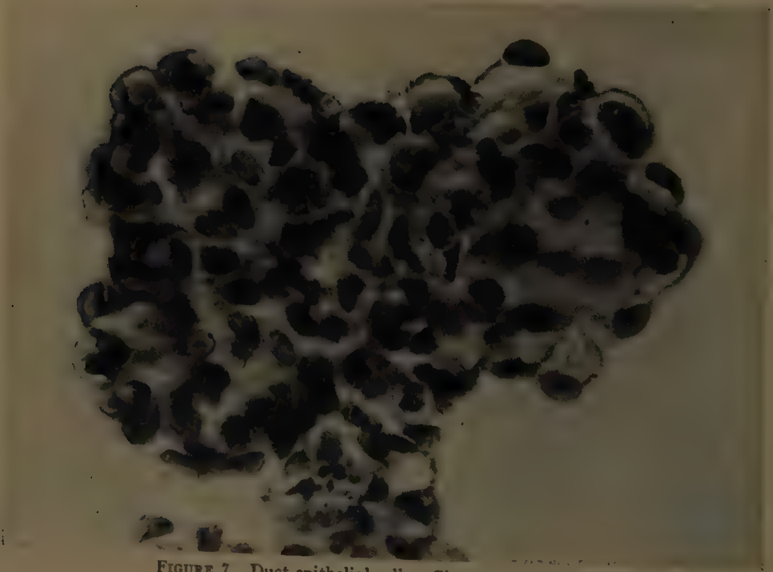


FIGURE 7. Duct epithelial cells. Cluster type C. ×600.

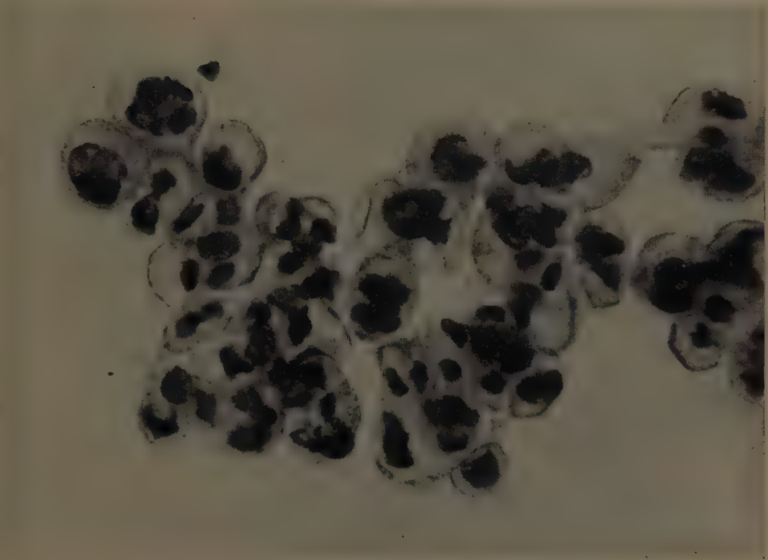


FIGURE 8. Duct epithelial cells. Cluster type D. ×600.

In type E (FIGURE 9), the cytoplasm dominates the picture. Each cell is swollen with one or more large vacuoles. The nuclei are apparently not increased in size over those in the previous three types and binucleation and multinucleation may occur. The nuclei are generally pushed to the cell periphery and distorted into a cup shape by the pressure of the vacuoles.

As previously stated, clusters of type A, that is, those with small undifferentiated cells, are sometimes noted in smears from apparently normal breasts of nonpregnant women. On the other hand, clusters of types B, C, D, and E might be interpreted as suggestive of intraductal papilloma because of their resemblance to the clusters seen in smears from this benign tumor (compare FIGURES 10 and 11).

The epithelial fragments desquamated during gestation or in the early postpartum period vary considerably in size irrespective of cell size. A few are very large and may be visible even with the naked eye (FIGURE 12). If judged by the criteria applied in the classification of breast smears from nonpregnant women, clusters of types B, C, D, and E show atypical features such as nuclear enlargement, binucleation and mulinucleation, cytoplasmic vacuoles, and some nuclear hyperchromasia. However, because of their presence bilaterally in breast secretion smears from a majority of the young women with clinically normal breasts in this series, it has been concluded that they are normal constituents of the mammary gland secretion during pregnancy and in the first week postpartum.

Epithelial cell clusters are most often seen in smears taken during the third trimester of pregnancy, with clusters of type B appearing in the highest fre-

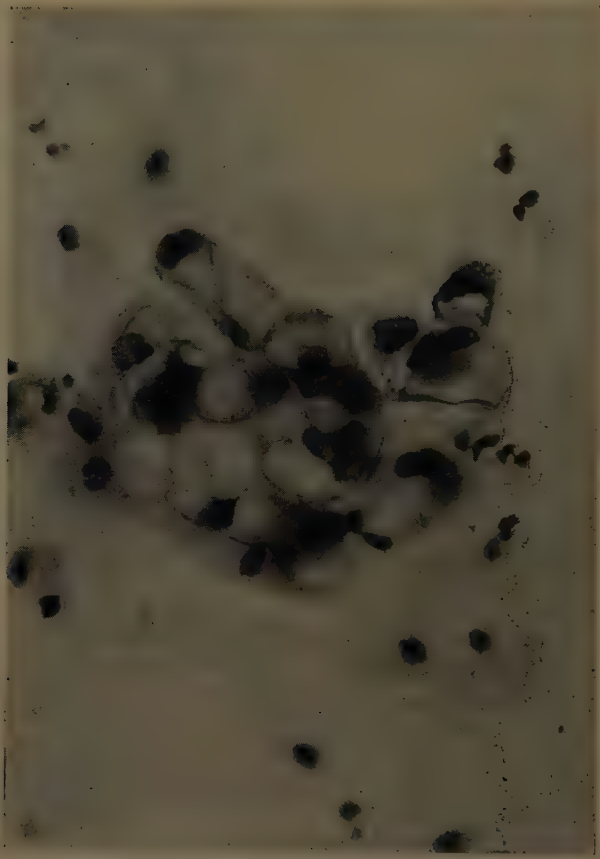
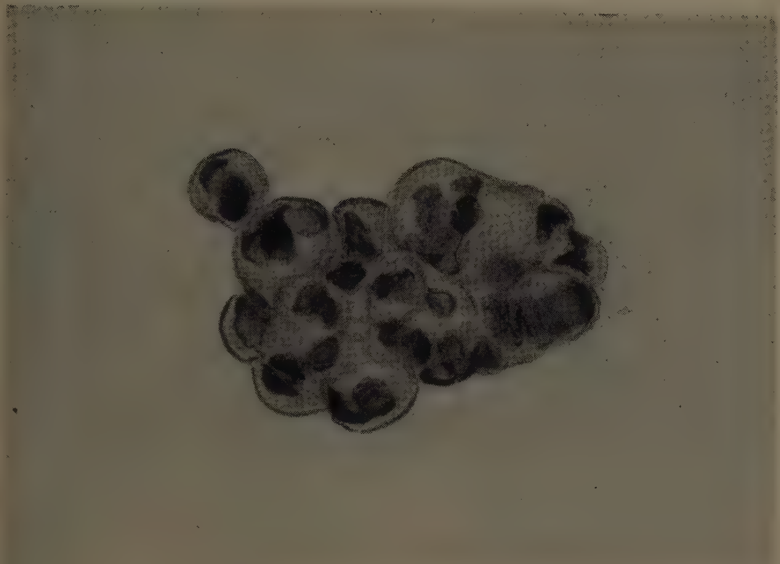


FIGURE 9. Duct epithelial cells. Cluster type E. ×600.

quency. During the first week following delivery, there are marked differences in the smears from women who are breast-feeding their infants and those who are given estrogens to supress lactation. In the former group, a vast majority of the smears are devoid of clusters and, in fact, may become acellular during the first postpartum week. A high percentage of the smears from women on estrogen therapy contain epithelial cell clusters of all the above-described types.

During the latter half of pregnancy and in the first postpartum week, the breast secretion smears may contain scattered single epithelial cells corresponding in morphologic type to the clusters of types B, C, D, and E. Undoubtedly, free cells of cluster type A occur, but they would be difficult to identify in smears because of their small size. There are also epithelial cells present that resemble those seen in alveoli in tissue sections of the mammary gland in the late antepartum period. Each of these cells contains an eccentrically located, well-preserved nucleus, and a single, large vacuole.



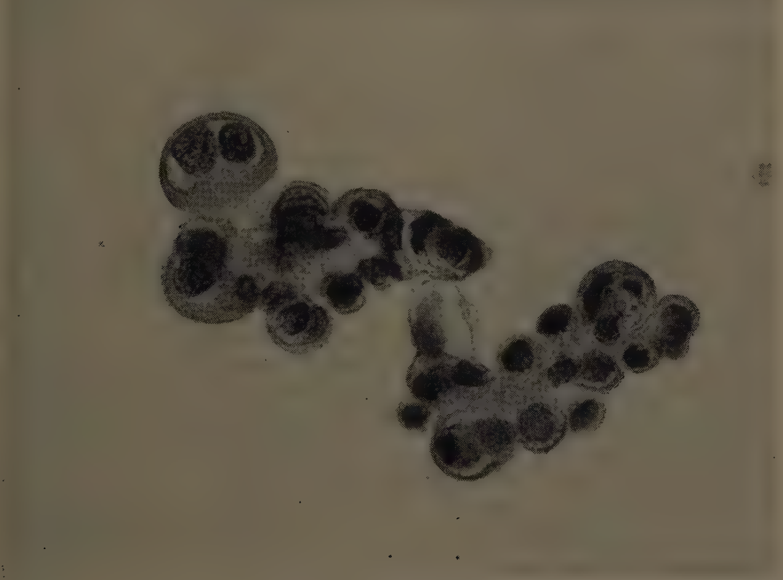


Figure 10. Cluster of duct epithelial cells from a breast secretion smear taken on the third postpartum day. ×600.

Figure 11. Cluster of cells found in a breast secretion smear from a postmenopausal woman. Pathologic diagnosis: intraductal papilloma. ×600.

Discussion

The common occurrence of papillary epithelial clusters in the breast secretion smears of pregnant and lactating women is difficult to explain on the basis of the usual histologic description of the breast during these periods (Berka, 1911;

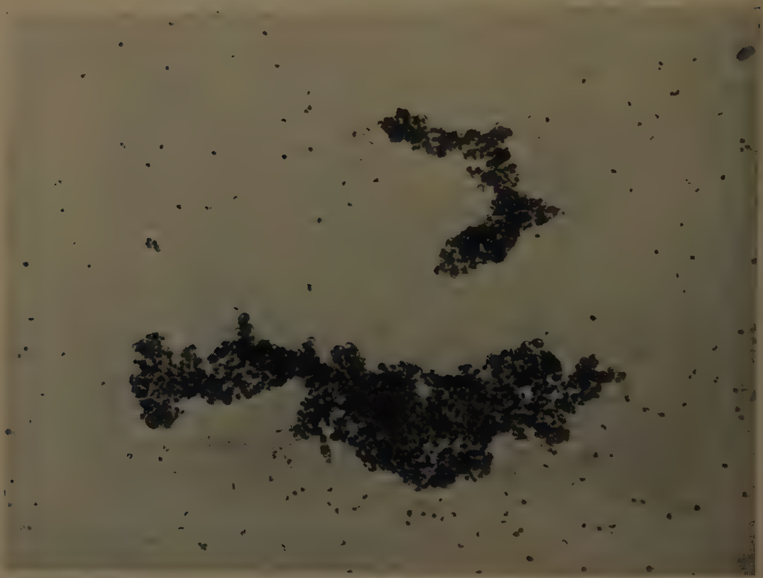


FIGURE 12. Two large clusters of duct epithelial cells found in a breast secretion smear taken during the third trimester of pregnancy. ×100.

von Eggeling, 1927; Loeb, 1932; Dawson, 1935; and Engel, 1953). The similarity of these clusters to desquamated fronds of intraductal papillomata suggests the possibility that the epithelium of the larger mammary ducts normally undergoes a micropapillary hyperplasia during pregnancy.

Our as-yet-incomplete review of the vast literature on the histology of the human mammary gland has not disclosed a description of such a micropapillary hyperplasia of the duct epithelium as a part of the normal mammary gland changes of pregnancy. A few relevant observations on lower mammals, however, have been found. Loeb and Hesselberg (1917) mention that in the proliferating stage of the mammary gland of the guinea pig "there may be papilla-like protrusions into the large ducts." Cole (1933) reported that in the mouse, after about seven days' lactation, the ducts develop fingerlike epithelial papillae that project into the lumina.

Other pertinent observations are to be found in reports on the response of the breast tissue of lower mammals and man to the administration of the sex hormones. Although mammary duct and alveolar growth have been described under various physiological and experimental conditions (for reviews, see Turner, 1932; Nelson, 1936; and Folley, 1952), a relatively small number of reports refer to the formation of epithelial papillae within the ducts. According to MacDonald (1936), estrin administration resulted in the formation of flat papillary projections of the mammary duct epithelium of ovariectomized rabbits. Eisen (1942) noted considerable hyperplasia of the duct epithelium with areas of the duct lumina filled with proliferating cells in rats given subcutaneous implants of estradiol dipropionate. Speert (1948) describes two castrated female Rhesus monkeys who developed intraductal epithelial proliferations resulting in microscopic papillomas after administration of amniotin followed by testosterone in one case, and testosterone alone in the second case.

In the human breast removed for cancer after estrogen administration of over two years' duration, Auchincloss and Haagensen (1940) found that the duct epithelium in the noncancerous portion of the gland was thrown up in low papillary folds. Similar findings were reported by Foote and Stewart (1945) in a postmenopausal woman who had been on stilbestrol therapy for one year prior to surgery. It is interesting to note that, in addition to the duct papillomatosis in this case, the noncancerous lobules of the gland were described as exactly duplicating lactating breast tissue. A rather high frequency of microscopic papillomatosis was observed by these authors, however, in both cancerous and noncancerous breasts of nonpregnant woman who were not receiving hormones.

Huseby and Thomas (1954) studied biopsies of the nondiseased portion of the mammary gland in a group of women before and after treatment with estrogenic hormones for advanced breast cancer. Microscopic examination of biopsies taken after therapy revealed, in some areas, a piling up of the duct epithelium that, according to the authors, was "not dissimilar to nonpedunculated ductal papillomas."

The above observations further support the concept that, in the human, papillary projections of the mammary duct epithelium may be formed normally during pregnancy and lactation in response to the complex hormonal stimuli of these periods.

The present investigation indicates that the smear technique affords an excellent method for following the cell changes in a single mammary gland throughout pregnancy and lactation. A serial histologic study is virtually impossible in the human, since frequent biopsy of the breast during the antepartum and postpartum periods seldom would be warranted. It is felt that the study of serial smears of human colostrum and milk may contribute to the understanding of cellular response to the complex hormonal influences normal to these periods and throw some light on reaction of mammary tissue to the therapeutic administration of hormones.

In regard to cytologic diagnosis, the present findings again stress the importance of a thorough knowledge of the physiologic variations of any organ as an essential basis for the evaluation of the abnormal. They emphasize further the desirability of having pertinent clinical data supplied with every diagnostic smear. A cytologist unfamiliar with the altered exfoliative cytology of the mammary gland during the antepartum and postpartum periods, or unaware that the patient under study is pregnant, might easily misinterpret the papillary epithelial clusters that appear to be normal to these periods as indicative of disease.

Thus far, we have not examined breast-secretion smears from pregnant women with mammary cancer. It seems probable, however, that malignant neoplasms could be detected in the latter half of pregnancy, or after parturition, when secretion is easily obtainable and epithelial desquamation appears to be heightened. It may be stated again, however, that extreme caution must be exercised in evaluating smears taken during these periods until we have gained a greater knowledge of the normal cytologic variation.

Summary and Conclusions

(1) Serial smears of human mammary gland secretion during the latter half of pregnancy and the first postpartum week were studied.

(2) Such smears contain foam cells, leukocytes, histiocytes, and single cells and cell clusters desquamated from the gland epithelium.

(3) Numerous epithelial cell clusters similar in configuration to desquamated fronds from intraductal papillomata were noted. The morphology of these clusters is described.

(4) It is suggested that the form of these epithelial cell clusters indicates that a micropapillary hyperplasia of the larger mammary ducts is normal to gestation.

(5) The possibility of utilizing breast-secretion smears during pregnancy and lactation in the detection of carcinoma is discussed.

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CYTOLOGIC-TISSUE CULTURE STUDIES ON CERVICAL EPITHELIUM*

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The human cervix is perhaps the best site for the study of cell changes in carcinogenesis. The main reason is the accessibility. The combination of the cytological smear and tissue culture methods is most ideal for observing cell changes, both *in vivo* and *in vitro*.[1]

The smear method permits one to study the cell behavior *in vivo* based on progressive morphologic cell changes.[2] It also permits the investigator to follow a specific case for a long period of time with serial cell studies. At the same time, small tissue fragments can be obtained for culturing.

One interesting application of the tissue culture method is the growth behavior of normal and malignant cells or the transitional changes occurring between normality and malignancy. Also, by this method, certain physiologic and morphological characteristics can be observed directly on the living cells.

Tissue grown *in vitro* has often contributed to differential diagnosis of doubtful tumors. In some cases, however, it is rather difficult to distinguish between normal and malignant cells. Malignant cells do migrate out of a tissue fragment much more readily than those of normal adult tissue, in which the growth may be scanty.

Mitosis is more common and more frequent in cultures of malignant tissues than in cultures of some normal adult tissues. Studies on the growth *in vitro* of human cervical tissues have been reported by several investigators.[3, 4, 5, 6]

This report deals with parallel studies of cell scrapings and tissue cultures made on 42 human uterine cervices, ranging from normal, inflammatory, precancer, cancer *in situ*, and clinical cancers. In some cases, five to six biopsied tissues were obtained from the same patient, taken over a period of months. In four cases, a four-winged instrument that divides the cervix into four segments was used,[6] permitting individual scrapings from each segment. At the same time, tissue fragments were taken from each segment for culturing. This method allows the localization of both the smear and tissue sampling, so that they can be compared with each other. In each instance, the punch biopsy method was used for obtaining the cervical tissues.

Materials and Methods

All the tissues were obtained under aseptic conditions. The patient was instructed to insert two vaginal suppositories containing 100,000 units of penicillin and 500 mg. of streptomycin the night before the operation. This was followed the next morning by a douche of 1:1000 Xephiran solution. In some cases, 1:250 Carlendacide solution† was used. As a douche, Carlendacide

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* These studies have been assisted by grants from the Damon Runyon Memorial Fund for Cancer Research, Inc., New York, N. Y.; the Public Health Foundation for Cancer and Blood Pressure Research, Inc., Stanford, Conn.; and the Davis Memorial Foundation, Inc., Wilmington, Del.

† Supplied by the Carlen Corporation, Coconut Grove, Fla.

contains several surface-acting materials that appear to be excellent for washing away the mucus and debris from the surface of the cervix and vagina. Immediately before the tissue is taken, the surface of the cervix is swabbed with 1:1000 Zephiran solution, followed by rinsing with sterile normal saline solution. The excised pieces of tissues were placed in sterile Ringer's solution containing 250 units per ml. of sodium penicillin and 5 mg. of streptomycin calcium chloride. When the segmental method was used for obtaining tissues, each piece was placed in an individual container and labeled. Immediately, the tissues were cut into fragments measuring 1 to 1.5 mm., at a right angle to the epithelial surface.

The Maximow double-coverslip method[7] was used throughout this study. The hanging-drop method is well adapted for high-power microscopic observation of living cells. A further advantage of this method is that, at the termination of the experiment, the single coverslip may be fixed and stained for critical cytologic studies.

The nutrient medium consisted of a mixture of one part fowl plasma, three parts human placental serum, and one part 50 per cent chick-embryo extract. To this mixture, the same amounts of antibiotics as above were added. Two fragments of tissue were planted on the coverslip with two drops of the above medium. From 30 and 60 fragments were planted in each case and were incubated at 37.5° C. Every 48 hours, the cultures were washed in Tyrode's solution, and a drop of fresh nutrient medium was added. No attempts were made to maintain the cultures for more than four or five weeks. At various times, cultures were fixed in Bouin's fluid and stained with hematoxylin and eosin for morphologic studies.

In every case prior to biopsy, careful serial cytologic smear studies were made and, in some instances, these studies extended over many weeks. The smears were taken by the Ayre surface cell-biopsy technique and were stained by the Papanicolaou method.

Observations

Normal. Smears show a typical normal picture. The cells are large, flat, water-shaped, some are precornified and basophilic, while others are cornified and acidophilic.

Cultures of normal cervical tissue showed the least growth and required a longer period of time for the squamous epithelial cells to appear beyond the edge of the explant. Only 10 per cent of the fragments showed cell migration over the average time of 10 days. In some cases, the outgrowing sheets appear to be from the cut edge at right angles to the outer layer of cervical epithelium, indicating that the cells may have migrated out of the basal layer. Each cell contained a single nucleus with little variation in size. Some cultures showed a few fibroblasts and leucocytes. In a few, there was some liquefaction of the plasma clot. Mitosis was never observed. After 10 days of incubation, no further outgrowths occurred. The culture remained quiescent over a period of five weeks, and the cells remained alone without any signs of degeneration. The rest of the cultures (90 per cent) showed only desquamated nonviable cornified cells. No morphological changes were noted in these cells.

Inflammatory. Smears from these cases showed a large number of leucocytes. Some of the epithelial cells showed a true cornification, but the majority showed evidence of irritation with hyperactivity of the nuclei and pseudocornification. The inflammatory cells appeared to have a tendency to universal acidophilia.

In cultures of tissues removed from cervices in which the smear showed typical chronic inflammation, the squamous epithelial outgrowths appeared to be richer than those seen in cultures of normal cervical tissues. Within 24 hours of incubation, a large number of leucocytes and a few histiocytes were seen. The epithelial cell outgrowths seemed to be richer than those observed in cultures of normal cervical tissues. In 25 per cent of the tissue fragments, epithelial sheets appeared beyond the edge of the explants. The average time for this outgrowth was 10 days, the same as that observed in normal tissues. The growth however, was more extensive. The cells exhibited some variation in nuclear size and a few were multinucleated. In some of the cultures, a few mitotic figures were seen, and the liquefaction of the clot was more extensive than those of normal tissues.

Precancer. In smears that show the precancer cell complex, there is a distinctive change in the morphologic character of the cell, showing incomplete differentiation and anaplastic changes. There was an increase in nuclear size with increased chromatin content. Many of the cells are multinucleated, and many of them may have a perinuclear halo. This halo may be so extensive as to give the cell a signet-ring appearance. The cytoplasm has a tendency to be stained acidophilic in color.

In cultures of tissues in which the precancer cell complex was present, 65 per cent of the fragments exhibit a luxuriant epithelial outgrowth within three days. The epithelial sheets extend far beyond the explant, much more than those seen in cultures of normal or inflammatory tissues. Many of the cells were multinucleated and irregular in size and shape, with prominent nucleoli. Some showed clear perinuclear halos with granulation of the cytoplasm. In some cases, the nuclei appeared to be free of cytoplasm or in various stages of cytoplasmic cytolysis. Mitotic figures were present in a large number of the preparations. The living cultures showed a greater cellular activity and greater liquefaction of the clot than appeared in cultures of normal or inflammatory tissues.

Cancer in situ. In smears from these cases, the cells exhibit pronounced morphological changes. The nuclei are much larger and are universally more active in appearance. There is a significant increased tendency to hyperchromatism. A majority of these cells are mononucleated, and they may appear in clumps or in small islands of three or more cells. The significant feature of these cells is that the nucleus does occupy a greater portion of the intact cytoplasm.

In cultures of tissues obtained from cases in which the cytologic smear showed cancer *in situ*, 75 per cent of the preparation exhibited extensive outgrowth of epithelial sheets within 48 hours of incubation. The cells within these sheets were contiguous and varying in size. A majority of the epithelial cells contained a single large nucleus occupying the greater portion of the cell. A few had a perinuclear halo, and some cells were multinucleated with prominent nucleoli. Mitosis was frequently seen in these cultures. The cellular activity and liquefaction of the culture medium was far greater than previously described.

Clinical cancer. In cervical scrapings taken from cases of clinical cancers, the cells vary in size. The nuclei are hyperchromatic and also vary in size and shape. The chromatin may be distributed evenly through the nuclei or it may be piled against the nuclear membrane, leaving a center that is less dense. The cytoplasm of some cells appears to be breaking down, reflecting the necrotic changes characteristic of clinical ulceration. The tumor cells, in most cases, are in clumps or in sheets several layers thick.

In cultures prepared from cervical cancer tissue, the outgrowth of squamous epithelium was marked in 90 per cent of the cultured fragments, making its appearance within 24 hours of incubation. Within the next 24 hours, the sheets extend far beyond the edge of the explant.

There is a great variation in size and shape of the cells and nuclei, and multinucleated epithelial cells were not as common as previously described. In a majority of the cultures, the outgrowing epithelial sheets were one-cell thick.

Spaces between the cells were present, showing the lack of cohesiveness between cells. The cells were otherwise contiguous. Mitotic cell division was present at all times and, in some cases, multinuclear division was observed. Histiocytes were present, but were not as marked as in cultures of normal, inflammatory precancer, and cancer *in situ* tissues.

The results observed in the experiments in which the four-wing instrument was used for dividing the cervix into four segments were of great interest. It was found that the type of growth seen in the cultures was a reflection of the cytologic grades seen in the smears. The more anaplastic the cells appeared in the smear, the greater was the growth activity in culture. In a single cervix, the cell types may vary from segment to segment. In one patient, it was found that her cells varied from normal to neoplastic.

Summary

The combination of the cytological smear and tissue culture methods for the study of pathological changes in human cervical tissue is unique.

Parallel studies using the two methods as a new approach to the problem of carcinogenisis have made possible not only the identification of characteristic features of cellular elements but, in addition, have provided a means of transmitting these characteristics to cultures of cervical tissues. The cytologic smear method presents cell behavior based on progressive morphological cell changes *in vivo*, while the tissue culture method provides a means for the study of physiologic and growth behavior of cells *in vitro*.

This investigation indicates that in tissue cultures of cases in which the cytologic smear shows cells of the precancer type, there is a marked activity of tissue growth that is midway between normal and clinical cancer. On the

other hand, cancer *in situ* behaves in tissue culture in the same way that clinical cancer does, except that its growth is slightly slower.

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EXFOLIATIVE CYTOLOGY AS APPLIED TO THE QUANTITATIVE EVALUATION OF ONCOLYSIS INDUCED BY VIRUSES

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The effect of neurotropic viruses upon transplantable mouse tumors was studied by Koprowski and Norton. These workers reported, in 1950, that each of these viruses destroyed or inhibited certain mouse tumors, multiplied without damage in others, and failed to multiply in some.[1]

Evaluation of the degree of oncolysis induced by a given virus in a solid transplantable tumor presented certain difficulties for following reasons. The accessibility of cells to viral infection varied in different portions of the tumor because of the presence of central necrosis. Small fragments of neoplastic tissue removed for histological examination and/or transplantation were therefore not sufficiently representative to judge the effect of the virus upon the tumor *in toto*. Furthermore, the understanding of the mechanism of the viral action suffered, since daily biopsies of the same animal were not feasible. At this early stage of investigation of oncolysis induced by viruses, the virologist was in search of an *in vivo* suspension of malignant cells similar to that observable in tissue culture techniques *in vitro*. He found such an *in vivo* suspension of malignant cells in ascites tumors of mouse.

One of the oldest ascites tumors of mouse, namely, Ehrlich ascites tumor, has been studied by numerous investigators in the past. George and Eva Klein, Goldberg, and Kurnick described some biological aspects of the use of ascites tumors.[2, 3, 4]

Ehrlich ascites tumor is characterized by a free proliferation of carcinoma in the peritoneal cavity of mouse and, in this respect, it is similar to human malignant neoplasms involving peritoneum. Malignant cells of this carcinoma have an increased value in the DNA content as compared to normal cells. Ehrlich ascites tumors maintained in different laboratories may vary in their chromosome numbers. The Ehrlich ascites tumor used in this study had a tetraploid modality of chromosome number.

From the standpoint of an exfoliative cytologist, Ehrlich ascites tumor offers excellent opportunities to study material that consists primarily of discr ete and uniformly distributed malignant cells. These cells are distinctly different from histiocytes, polymorphonuclear leukocytes, and lymphocytes, which are also present in smears from perinoneal aspirates. In those circumstances, qualitative changes in cells, as well as variations in differential cell counts, may be evaluated easily and accurately in the course of studies of the effect of various agents upon this tumor.

Preliminary morphologic studies of uncontaminated Ehrlich ascites tumors obtained in 1950 from the Carlsberg Foundation in Copenhagen, Denmark, were carried out at Papanicolaou Cytology Laboratory at Cornell University Medical College in New York, N. Y. Serial passages of tumors in Swiss Albino and Strong A mice were accomplished by aspirating several cubic centimeters of bloody peritoneal fluid from tumor-bearing mice and injecting new mice intraperitoneally with a few tenths of 1 cc. Smears were prepared from each aspirate used for passage by being fixed in ether alcohol and then stained by Papanicolaou's technique and examined. The examination consisted of a qualitative appraisal of characteristics of malignant cells and of a quantitative evaluation of their proportion by means of differential counts.

Usually malignant cells represented as much as 80 per cent of the cells examined, and they were characterized by large hyperchromatic nuclei with prominent nucleoli. The cytoplasm was often vacuolated and multinucleation was common. The malignant cells were fairly uniform in size and shape. Nonmalignant cells consisted of histiocytes, polymorphonuclear leukocytes, and lymphocytes. Mesothelial cells could be distinguished from histiocytes only in rare instances. Erythrocytes were commonly present. Mitoses predominantly occurring in malignant cells were common. The most readily observable abnormalities of the mitotic mechanism consisted of multipolar divisions.

Following preliminary morphologic study of the uncontaminated tumors, the mice were transferred to Doctor H. Koprowski's laboratory at the Viral and Rickettsial Division of the Lederle Laboratories, Pearl River, N. Y., where large groups of tumor-bearing animals were inoculated with viral agents, usually four days after implantation. Peritoneal fluids were aspirated from virus-infected mice and from noninfected control animals. Smears were prepared from pools of aspirates from the same experimental groups of animals and delivered in ether alcohol to the Papanicolaou Laboratory for cytologic studies. The results of that study were described elsewhere in detail.[5] By comparing differential counts of cells in smears prepared from virus-infected fluids with those in smears from noninfected control fluids, 13 viruses were investigated as to their tumor-inhibiting activity. The simplicity of this method may be illustrated best by following examples of differential counts.

In the case of Eastern E.E. virus (which does not proliferate in cells of the Ehrlich ascites tumor nor interfere with the neoplastic process), the following differential count was obtained in one of the early experiments: 73 per cent cancer cells, 7 per cent histiocytes, 11 per cent polys, and 9 per cent lymphocytes, as compared to 72 per cent cancer cells, 19 per cent histiocytes, 2 per cent polys, and 7 per cent lymphocytes in the case of the corresponding control smear. In the count of a typical experiment with Bunyamwera virus, the oncolytic properties of which were proved conclusively by this method, the number of cancer cells dropped to 3 per cent (47 per cent histiocytes, 4 per cent polys, 46 per cent lymphocytes) as compared to 76 per cent cancer cells, 16 per cent histiocytes, 4 per cent polys, and 4 per cent lymphocytes in control slides.

These two viruses EEE and Bunyamwera represent, respectively, extreme examples of complete lack of a tumor-inhibiting activity and of a very powerful antitumor activity.

Some of the examined viral agents did not give immediately clean-cut results. One of these (Mengo virus) became the subject of a separate communication.[6]

In the case of Mengo virus, the transplants prepared from infected ascites tumor cells invariably caused death of the animals from viral infection, so that the capacity of the virus-infected neoplastic cells to produce tumors when injected subcutaneously into mice could not be assayed. The cytologic evidence, however, yielded convincing data as to the marked oncolytic property of this virus. Some interesting features of oncolysis were noted in the daily observations of smears obtained from individual animals.

Destruction of cancer cells with the use of the more concentrated viral inocula was usually accompanied by disappearance of mitoses from corresponding smears. As a rule, this did not occur in mice in which oncolysis was induced by higher dilutions of virus. On the contrary, it was noted that in some cases the number of mitoses exceeded that observed in the cells of noninfected ascitic fluid. Although abnormal mitotic figures were seen in malignant cells, normal mitoses were observed also in degenerated cells, and it was uncertain whether the latter were of neoplastic or histiocytic origin.

Smears in which cancer cells were reduced in number usually showed a coincident simultaneous increase of cells in karyorrhexis. It may be important to note, however, that not all the cells in karyorrhexis were of the malignant variety.

Another observation concerns variations in the number of cancer cells encountered in smears of ascites fluids from different animals inoculated with the same dilution of virus. In several mice, the ultimate cell count failed to indicate a marked decrease in the number of cancer cells as the result of infection. A definite decrease in neoplastic cells, however, was found in counts on other animals inoculated with the same dilution of virus. This might be explained by the assumption that a decrease of cancer cells could have occurred during the time elapsing between the last cell count and the death of the animal. It is also possible that some mice died of infection before oncolysis took place.

In control animals, variations in the percentage of cancer cells were only minimal. Nevertheless, in two control mice, cancer cells were as low as 33 per cent and 25 per cent. The 33 per cent cell count was transient and was followed by a high count on the following day. It might have been due also to an experimental error. The 25 per cent of malignant cells in a control smear may possibly indicate that in rare instances exfoliation of cancer cells can be reduced even in untreated mice.

The unusually shaped histiocytes observed in this study were not seen in control smears of mouse ascites tumors. They resemble "epithelioid" cells present in sections of tissue from the granulomatous type of lesions. It appears likely that the transformation of histiocytes into "epithelioid" cells may occur in the ascitic fluid of mice, and that it results from ingestion of large quantities of phagocytized material.

In the case of the West Nile virus, no cytologic evidence of oncolysis was obtained in Swiss Albino and Strong A mice. The oncolytic action of this agent, however, was demonstrated morphologically in PRI mice.

The PRI strain of mice was observed by Sabin to be genetically resistant to the lethal effect of West Nile virus through interference with its propagation. The Egypt 101 strain of West Nile virus was injected by Doctor H. Koprowski into PRI mice previously inoculated with ascites tumor cells. While all noninfected animals died from tumors within 11 to 33 days after implantation, only a small number of the virus-infected mice died from ascites tumor, and their death occurred later than in the control animals. These data support the findings that West Nile virus exercises a biologically discernible interference with neoplastic process.

The West Nile virus has been used at Memorial Center for Cancer and Allied Diseases in New York, N. Y., for the experimental treatment of patients with advanced metastatic and inoperable cancer.[7, 8] The quantitative evaluation of a possible oncolytic effect of this agent upon the exfoliated cells in pleural and peritoneal fluids of human patients was applied to the clinical material obtained from the Memorial Center.[9] The quantitative evaluation of human serous effusions, however, is applicable only to selected cases, because of the following reasons:

(1) If the number of malignant cells is very small prior to the induced infection, a demonstration of a significant decrease following treatment may be impossible.

(2) Malignant cells are often grouped in irregularly scattered clusters, the presence of which results in a great variation in differential counts made on different portions of the slides.

(3) Differentiation of malignant cells from dubious atypical cells that may be of histiocytic or mesothelial origin can be very difficult or impossible.

(4) It was observed in the course of this study that neither right and left pleural effusions nor pleural and peritoneal fluids are comparable in the same patient. Therefore, only differential counts made in smears of serial samples of fluids from the same source should be compared. One wonders whether there may be a correlation between different cell counts and different types of cells in pleural and peritoneal fluids of the same patients, as shown in this study, and different chromosome numbers in malignant cells seen in pleural and peritoneal fluids of the same patient, as recently reported by Koller.[10]

As to the results of this study in humans, there was no morphologic evidence, in the six cases examined, that the Egypt 101 strain of West Nile virus had any definite oncolytic action. In two cases, the percentage of malignant cells became lower following the infection and some striking karyorrhectic phenomena were observed. In four of five cases of carcinoma the lymphocytes became more numerous in the serous effusions following the induced infection. In a single case of lymphosarcoma, the lymphocytes presumbably representing neoplastic elements, yet morphologically indistinguishable from normal lymphocytes, became less numerous subsequently to inoculation of virus. The difficulty in distinguishing neoplastic from nonneoplastic lymphocytes was also encountered when exfoliative cytology technique was applied to study of contact smears of human lymph nodes.[11]

In a special study carried by the author since 1950 in cooperation with Doctors Engle and Ultmann from the Department of Medicine at Cornell University Medical College, efforts were made to distinguish malignant from nonneoplastic lymphocytes in a series of contact smears of lymph nodes.[13, 14] Diagnoses of intrinsic neoplasms usually have been made correctly in those smears in cases of lymphomas characterized by a predominance of immature lymphocytes or reticulum cells, and in cases of Hodgkin's disease with presence of Reed-Sternberg cells. In cases of lymphocytic leukemia, lymphosarcoma of the lymphocytic type (small cell), and giant follicular lymphoma, the diagnoses, when correct, were based upon the proportion of various types of cells in smear rather than upon conclusively abnormal features of mature lymphocytes.

The application of exfoliative cytology to the quantitative evaluation of oncolysis induced by viruses has been of value to virologists as well as cytologists.

The use of exfoliative cytology contributed data for a reclassification of neurotropic viruses on the basis of their action upon cells of the ascites tumor and provided evidence of the oncolytic action of several viruses. It helped to elucidate the mechanism of oncolytic action of some viruses by studying daily aspirates from tumor-bearing infected animals. Since virologists presently use ascites tumors for propagation of viruses, exfoliative cytology continues in its usefulness in the evaluation of tumor-destroying properties of viral agents.

The advantage to a cytologist devoting his interests to studies of a limited number of virus-infected tumors may not still be apparent to many. The broadening of the scope of exfoliative cytology by utilizing it as a tool in a variety of branches of medical research is a reward in itself. We have to consider also that the present day exfoliative cytology is no longer a purely qualitative science. Inasmuch as a clinician is still primarily interested in knowing whether or not malignant cells are present in patient's secretion submitted for diagnostic evaluation, he begins to appreciate quantitative data. He is interested in knowing whether the cytologic findings are suggestive of a definite type of a malignant neoplasm, such as epidermoid carcinoma, adenocarcinoma, anaplastic carcinoma, tumor of the lymphoid type, *etc.* The gynecologist asks whether the epidermoid carcinoma of the cervix is an early or an advanced one. The radiotherapist asks whether the tumor is radiosensitive or not, and what is the effect of his therapy upon exfoliated cells. Differential counts have been used by Ruth Graham in vaginal smears of patients with carcinoma of the cervix for the evaluation of the sensitization reaction and of the effect of the irradiation therapy.[12] Differential counts in smears from pleural and peritoneal fluids of inoperable cancer patients subjected to induced virus infections have been mentioned already. But in smears from human secretions and fluids, no treatment, thus far, has been shown to induce as clean-cut and easy-to-evaluate variations in differential counts as are obtained in smears from peritoneal fluids of ascites tumor-bearing mice subjected to oncolytic agents. The availability of this excellent type of reference material is of a particular value to cytologists who wish to base their evaluation of the effects of any therapy upon variation in cellular counts.

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STUDIES ON NUCLEOPROTEIN PATTERNS IN RADIOSENSITIVITY TESTING*

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The clinical background for these radiosensitivity studies in gynecology can be understood best by noting the revival of radical hysterectomy as a mode of treatment for cancer of the cervix and its recent establishment as a therapy which may, in operable stages of the disease, offer a result fairly comparable to that obtained with radiation. Efforts to make these methods of treatment complementary rather than competitive have provoked much interest in studies that have attempted to define and codify tissue changes under radiation.

The problem of radiation sensitivity of human cervical cancer has many facets, most of them relatively poorly understood. We know, for example, that the majority of such tumors are grossly radiosensitive, for the primary site will heal in more than 90 per cent of cases, even in instances where the patient dies of lateral parametrial spread or distant metastases. Thus, the matter of relative sensitivity must be considered in an effort to understand better the role of these many factors, which must be important in healing:

(1) Variation in dosage with different radiation techniques, some inadequate to deal with lateral disease in parametria and nodes, and even centrally inadequate, at times with anatomic distortion by the tumor.

(2) Variations in response with varying tumor differentiation and lack of homogeneity in any tumor population.

(3) The possible variation in response of exophytic or endophytic tumors and its relation to the quality of the tumor bed or stroma. Certainly, the reaction of the stroma to radiation must be as important in the containment of a tumor as is the direct attack and destruction of cells by gamma and X rays.

(4) The general health and habitus of the host, which seems to affect healing in a poorly understood manner.

(5) The local cauterization effects of radium, which may be unrelated to the more subtle direct action of gamma rays on tumor cell growth and multiplication. It is important to note that the uterus is unique amongst bodily organs in its favorable configuration as a radium receptacle.

(6) The possible variation in sensitivity of tumor cells in lymph nodes from that of the primary cervical tumor.

Our interest in studying cytochemical patterns of tumor cells under radiation arose from the belief that such investigations of their nucleoprotein patterns might not only offer some explanation for the morphologic changes described in the past, but might also offer us a more tangible approach to clinical testing, by the definition of changes gross enough to be translated into the clinical techniques of workaday pathologic laboratories. We were attracted further to

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* The work described in this paper was supported by Grant No. C1793 of the United States Public Health Service, Bethesda, Md.

the study of cytochemical changes by the work of Caspersson,[1] who demonstrated by the aid of ultraviolet spectrophotometry that there were two types of tumor cells: A cells with nuclei heavy in chromatin pattern, thus rich in DNA, and B cells, so-called, with nuclei relatively poor in chromatin DNA, but with prominent nucleoli rich in RNA. The A cells were noted to be vital, aggressive cells, found around blood vessels and in the growing zone of the tumor. The

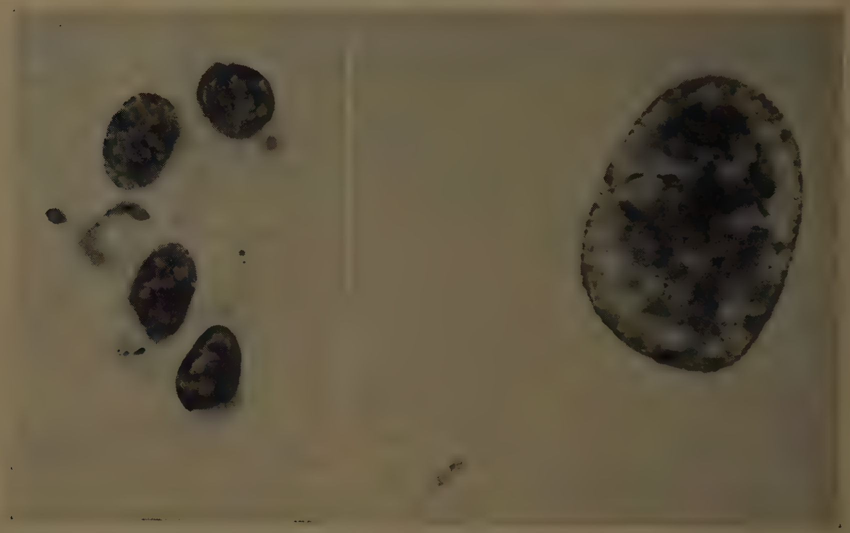

FIGURE 1. Oil-A.S.-orcein-fast green technique. To the left is a group of A cell nuclei, preradiation. To the right is an R cell nucleus, after onset of radiation therapy. ×918.

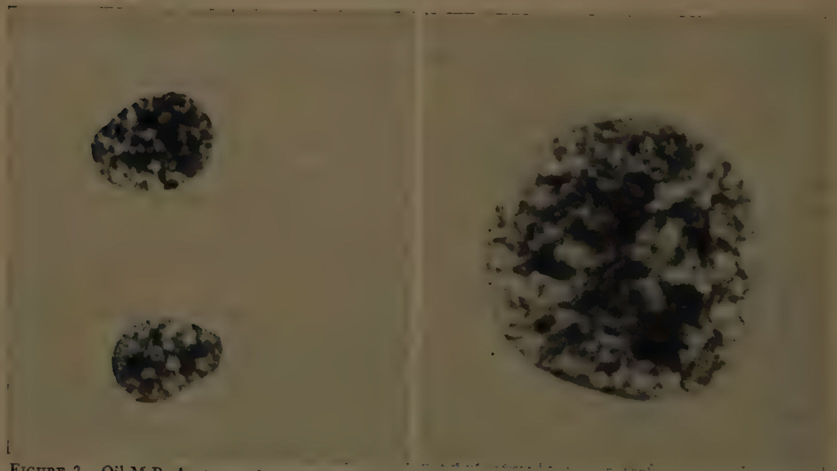

FIGURE 2. Oil-M.B.-Acetocarmine technique.[5] To the left are a group of A cells (nuclei) before radiation. Note heavily clumped chromatin material. To the right is an R cell with much larger nucleus, and beginning diffusion of chromatin. Nucleoli are more prominent. This change has occurred after radium application. ×918.

B cells were considered regressive, prenecrotic cells found in abundance in the central, often soft part of the tumor.

We developed our studies with the thesis that the growing edge of a cervical cancer prior to radiation should be rich in A cells, and that this zone might show a conversion to a predominance of B cells under radiation if it were properly responding by healing.

Methods

We obtain quadrant biopsies from the growing active zone of the tumor before, and at weekly intervals during, radiation. Our radiation method is a modified Curie technique of radium application usually delivered in one sitting of 100 hours, followed by external X-ray therapy. Because the destruction of the primary tumor is so dramatic with radium, nuclear changes are better studied under the gentler healing with external X-ray therapy. Our preference, clinically, for the radium application first, however, and the knowledge that external radiation is difficult to standardize from the point of view of specific

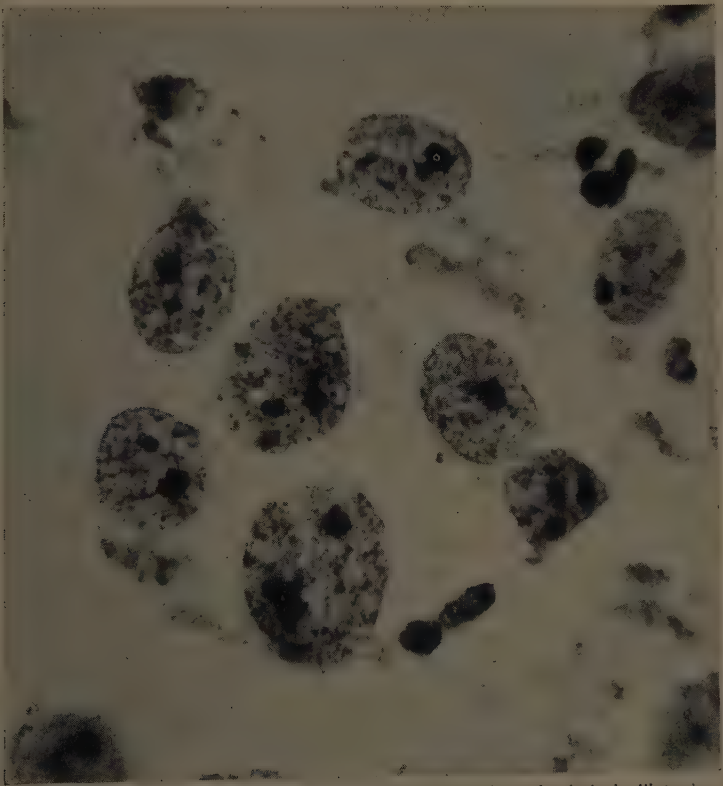

FIGURE 3. Oil-A.S.-Acetocarmine technique. A group of B cell nuclei with relatively dilute chromatin and prominent large and frequently multiple nucleoli. ×918.

doses at specific biopsy sites has led us to the use of a preliminary test dose administered by transvaginal cone. At the moment, this test dosage is 2000 r in air.

The quadrant biopsies are kept moist in physiological saline, and a portion of each used for sections stained by conventional H and E and also by methyl-green pyronin techniques. These sections are used for general survey controls. Tissue smears are now made from each biopsy and stained by orcein-fast green[2] and methyl green-pyronin techniques.[3] Upon these smears, which furnish ex-

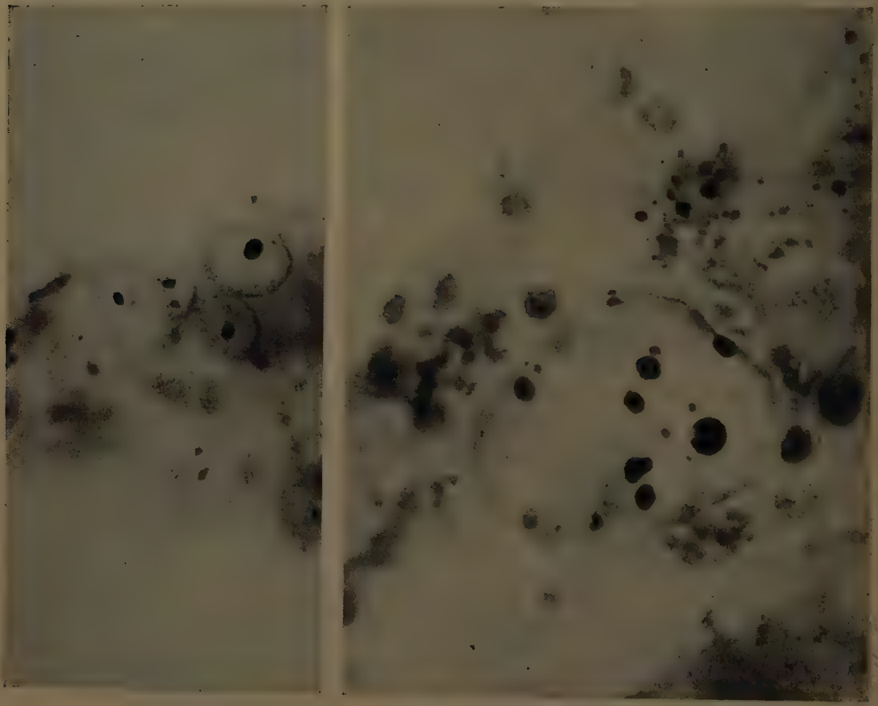

FIGURE 4. Oil-Methyl-green-pyronin technique. Prior to radiation on the left, small nucleoli are seen. In this group, the chromatin is poorly seen. To the right is a huge radiated nucleus with multiple large nucleoli ×918.

TABLE 1

RADIOSENSITIVITY TESTING—CYTOLOGIC

Stage	Total	Good	Fair	Poor
I	9	6	2	1
II	24	19	3	2
III	11	8	3	0
IV	1	0	0	1
Totals	45	33	8	4

In all tables, cytologic refers to acetocarmine[5] or orcein-fast-green techniques. Cytochemical refers to the methyl green-pyronin method.

cellent nuclear definition, cell population counts are made before, during, and after radiation. These two staining techniques are complementary in that the first has been found superior for the definition of chromatin patterns (DNA) and for the observation of morphologic differences that enable one to distinguish

TABLE 2
RADIOSENSITIVITY TESTING

Cytologic response		*Good: 33 cases*
Clinical response:		
Good	29	
Fair	4	
Poor	0	
Cytologic response		*Fair: 8 cases*
Clinical response:		
Fair	6	
Poor	2	
Good	0	
Cytologic response		*Poor: 4 cases*
Clinical response:		
Poor	4	
Fair	0	
Good	0	

Four patients were reported "good" cytologically but "fair" clinically. Three were in Stage II; one was in Stage III. The cervix healed in all cases. All died, two with bone metastases, one with lung metastases, one with peritoneal metastases. Two patients were reported "fair" cytologically, but "poor" clinically. One was in Stage II, one was in Stage III. The cervix never healed in either case. Both patients are dead.

TABLE 3
RADIOSENSITIVITY TESTING

Cytochemical response		*Good: 15 cases*
Clinical response:		
Good	13	
Fair	1	
Poor	1	
Cytochemical response		*Fair: 2 cases*
Clinical response:		
Fair	1	
Poor	0	
Good	1	
Cytochemical response		*Poor: 4 cases*
Clinical response:		
Poor	1	
Fair	2	
Good	1	

One patient was reported "good" cytochemically, but "fair" clinically. She was in Stage II. The cervix healed but she died with bone metastases. One patient was reported "good" cytochemically, but "poor" clinically. This patient was also in Stage II. She died, the cervix having never healed. One patient was reported "fair" cytochemically but "good" clinically. This patient was in Stage I. The cervix healed. She died of pulmonary emboli. Autopsy revealed no cancer anywhere. Two patients were reported "poor" cytochemically but "fair" clinically. Both were in Stage II. One showed slow but eventual healing of the cervix. Lymph node dissection was not done. The other showed healing of the cervix but one lymph node was positive. One patient was reported "poor" cytochemically but "good" clinically. This patient was in Stage III. The tumor regressed strikingly under supervoltage radiotherapy. The cervix healed and, when last seen, there was no demonstrable tumor anywhere. The patient died of unknown cause in another city, about seven months after completion of radiotherapy.

TABLE 4
RADIOSENSITIVITY TESTING—15 CASES STUDIED IN PARALLEL

Patient	Cytologic	Cytochemical	Clinical
No. 29—L. C.	*Good*	*Good*	*Good*
No. 31—E. G.	*Fair*	*Fair*	*Fair*
No. 34—A. P.	*Good*	*Good*	*Good*
No. 35—I. B.	*Good*	*Good*	*Good*
No. 36—A. B.	*Good*	*Good*	*Good*
No. 41—K. B.	Fair	Good	Poor
No. 42—M. D.	*Good*	*Good*	*Good*
No. 43—A. S.	*Poor*	*Poor*	*Poor*
No. 50—M. H.	*Good*	*Good*	*Good*
No. 54—M. A.	Fair	Poor	Fair
No. 56—V. K.	Good	Good	Fair
No. 57—M. D.	*Good*	*Good*	*Good*
No. 61—B. T.	Good	Poor	Good
No. 64—L. C.	*Good*	*Good*	*Good*
No. 67—B. N.	*Good*	*Good*	*Good*

No. 41. K. B., Stage II. Cervix never healed. Patient died of tumor.
No. 54. M. A., Stage II. Cervix healed slowly. Patient alive and well.
No. 56. V. K., Stage II. Cervix healed, bone metastases. Patient died of tumor.
No. 61. B. T., Stage III. Cervix healed. Patient was alive and well when last seen. Died of intercurrent disease.

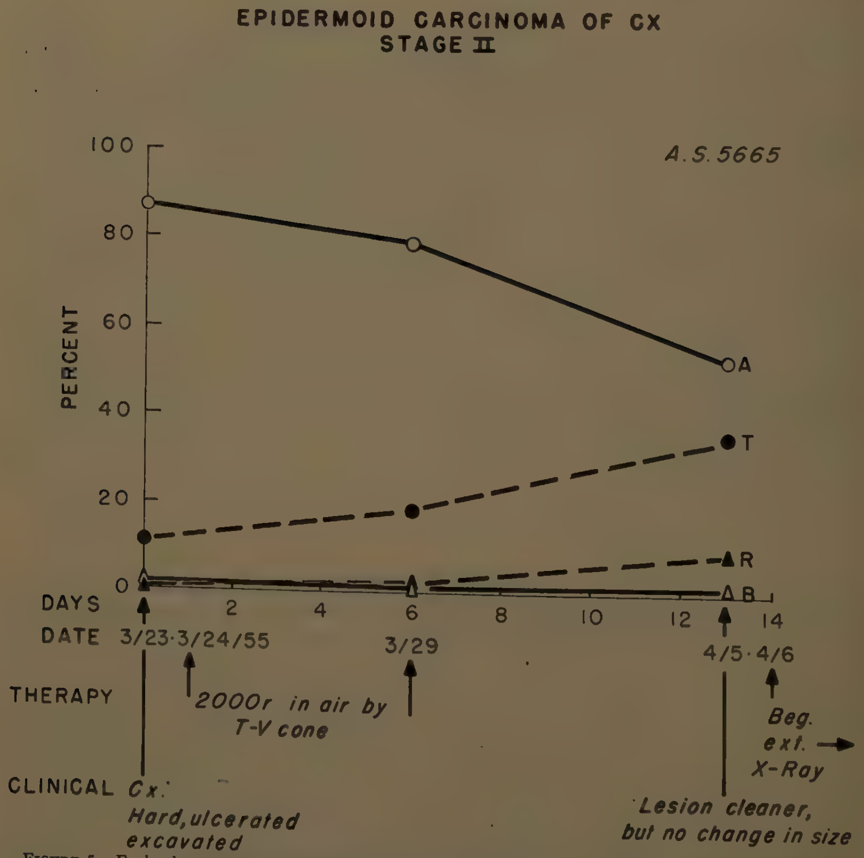

FIGURE 5. Early change with small test dose of radiation. Decline in A cells and rise in T & R cells is beginning.

malignant cells, while the latter gives greater cytochemical specificity for DNA-RNA estimation and is especially useful in the identification and study of nucleoli (RNA). Both are differential stains to some extent, for the orcein and the methyl green are chromatin stains, while fast green and pyronin are better nucleolar stains.

The cell counts are made on the orcein-fast green smears, with four general tumor cell types defined as follows: A cells, with a nucleus heavy in chromatin material and a relatively obscure nucleolus; B cells, whose much larger nuclei have a diffuse, poorly defined chromatin pattern but a prominent nucleolus or nucleoli; T cells, intermediate between these categories; and R cells, which have retained an A type of chromatin pattern yet show radiation reaction characterized by enlargement of the nucleus and, sometimes, by vacuolization (FIGURES 1, 2, and 3).

The greater specificity of the supplementary methyl green-pyronin technique enables one to characterize the highly polymerized DNA of the chromatin more precisely and brings the definition of the nucleolar material to a point where their diameters can be measured, thus furnishing a rough RNA assay (FIGURE 4).

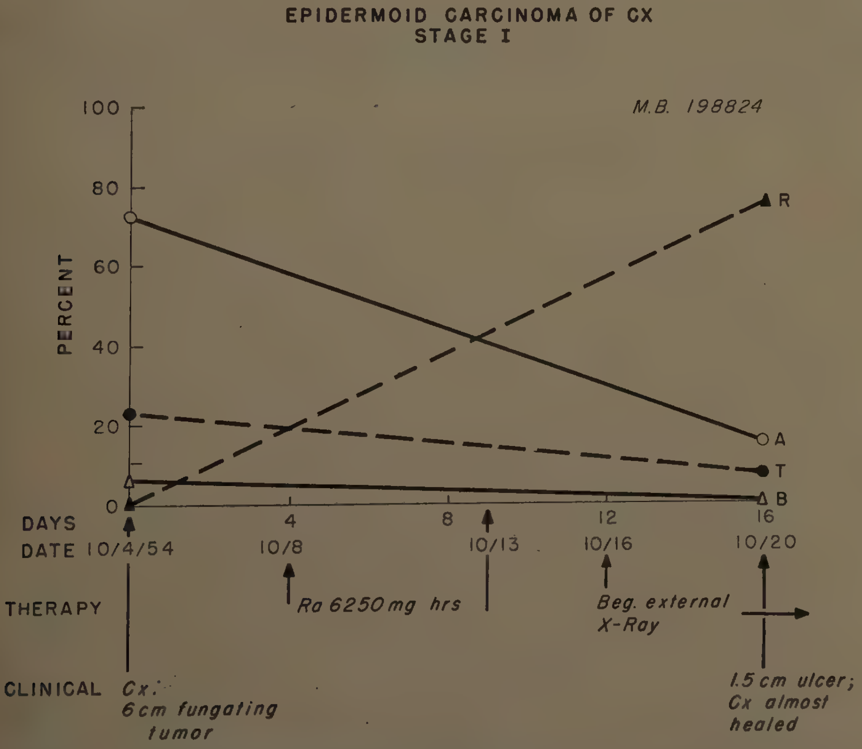

FIGURE 6. Good response, though B cells have not risen notably as yet. Excellent decline in A, and rise in R cells.

We have not been impressed with any detriment to the healing of these tumors by serial biopsy sampling as suggested by others.

Results

Our studies suggest that healing of human cervix cancer can be predicted with some uniformity from the observation of nuclear changes occurring within two to three weeks after the onset of radiation in the growing, vital zone of the tumor. The observed changes include: (1) enlargement of the cell and its nucleus; (2) a relative diminution in highly polymerized DNA evidenced by a sort of dilution of the chromatin masses; and (3) a relative increase in RNA as evidenced by an increase in nucleolar size and number. These changes are estimated qualitatively, but they have been made semiquantitative by the cell population counting system and measurements of nucleolar diameters.

In summary, one might state that we have been enabled to predict tumor healing if there has been a shift away from a predominance of an A cell, or active phase, in the growing zone, toward a B cell, or regressive-phase, emphasis or, expressed in another way, an alteration in the DNA/RNA ratio with a relative shift toward RNA predominance. It is true that these changes are relative, for it would appear that the RNA seems to increase, in general, at about the same rate as does the general cell and nuclear volume. The DNA, on the other hand, probably changes very little and therefore shows a relative decrease in

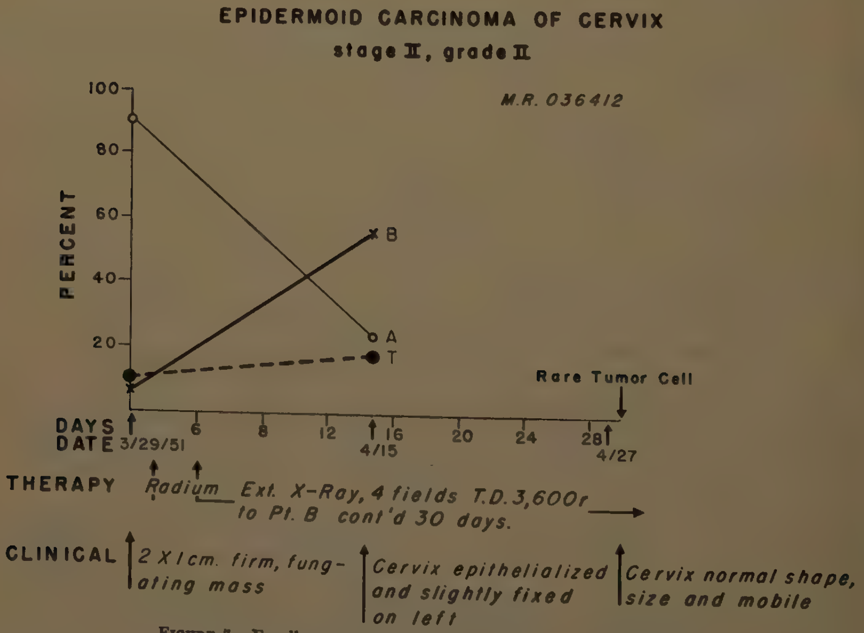

FIGURE 7. Excellent response with reversal of A and B cell counts.

concentration. This would suggest a specific effect of irradiation on the synthesis of DNA.[4]

As we have demonstrated in TABLES 1 to 4, our clinical correlation has been good. The number of our cases is small, in spite of the five-year tenure of our work, because we have experimented with staining methods, and because our increasing experience has forced us to discard atypical tumors with questionable reactions. It seems that the longer we work, the fewer cases we admit to the body of our valid data. We are now making renewed efforts to standardize our reactions by the test-dose technique. It is important to understand that we have been interested principally in correlation with healing of the primary tumor. If this occurs, it is labeled a good response. If the primary tumor heals, but a lateral parametrial, lymph node, or distant metastasis occurs, we call this a fair clinical response. Only failure of the primary lesion to heal is considered a poor response. We have avoided the term radiocurability for the present because of the numerous other factors that may be involved in addition to pure cell sensitivity.

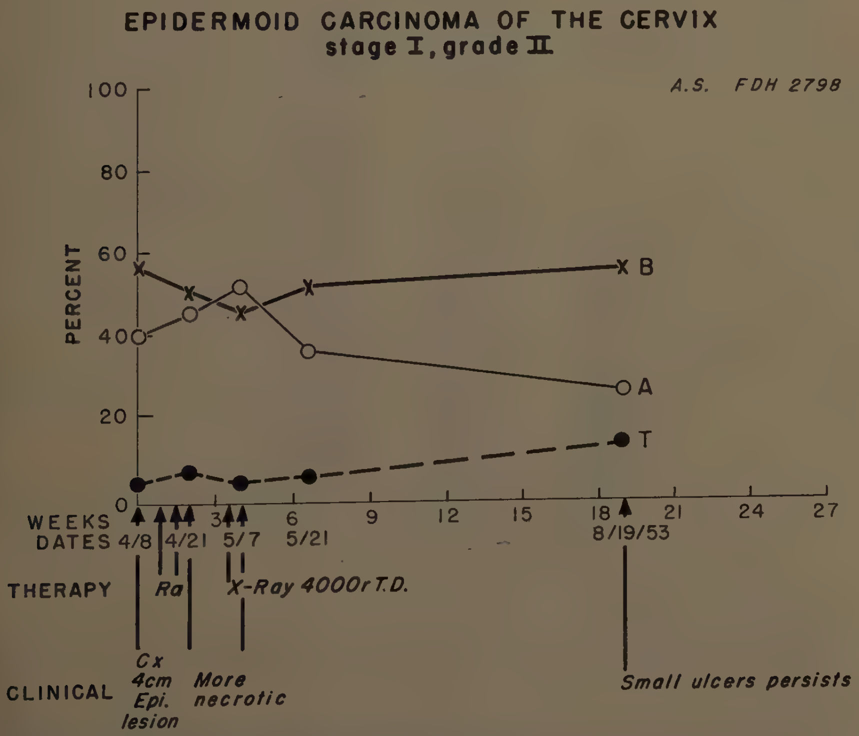

FIGURE 8. Lack of notable cytologic response in a radioresistant tumor. Slight B cell predominance of unknown significance, but lack of change is very important.

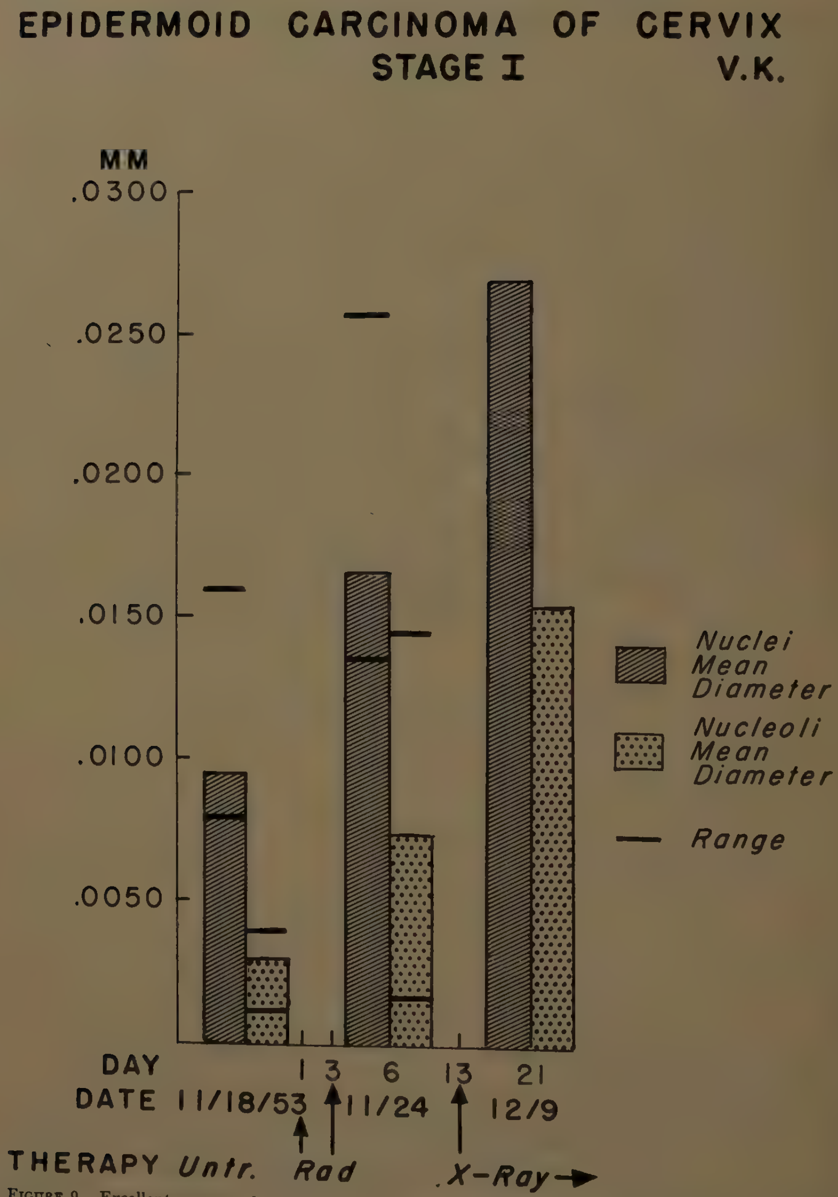

FIGURE 9. Excellent response by methyl-green pyronin technique, with increasing nuclear and nucleolar diameters.

Discussion

It is our hope that such cytochemical studies of nucleoprotein patterns will enable us to predict tumor cell death in a uniform fashion before conventional histologic or clinical methods can detect it. This would be a major adjuvant in the management of patients with cervical cancer, and could help us enormously in selecting patients for radiation or surgery, or for a combination of these methods of treatment.

We believe that the principles presented are valid and that a shift in cell population may be expected in a radiosensitive tumor under radiation from a predominance of the vital, aggressive tumor cells characterized by a heavy DNA-chromatin configuration to a pattern characterized by relatively inactive cells with a relative decrease in DNA and a relative increase in nucleolar RNA. It is not our conviction that the techniques we have used are necessarily the most suitable cytochemical ones for this approach. The evidence does seem to support the belief that such a nucleoprotein shift does occur and may be utilizable for radiosensitivity testing in this area.

These methods differ in some respects from others used for similar analyses, but it would appear that they may all share certain basic concepts.

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THE SENSITIZATION RESPONSE IN THE VAGINAL SMEAR*

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Some nonmalignant epithelial cells of the vaginal smear have a distinctive appearance: the cytoplasm is dense, it stains basophilic, and it is finely vacuolated. The presence of these cells indicates the sensitization response, or SR. The least differentiated, so-called "basal" cells, that come from the deeper layers of the epithelium, are the ones that ordinarily exhibit SR, but occasionally it is seen in the more superficial "precornified" cells as well. This picture is found in varying intensity and frequency. Our efforts to measure SR have been confined to an expression of the percentage of cells exhibiting it; *i.e.*, the result of a differential count on 100 consecutive nonmalignant epithelial cells. The changes are sufficiently definite to enable observers trained in cytology to distinguish SR in a brief period, if the material is properly prepared. SR can be demonstrated with a variety of stains and fixatives. For proper evaluation, however, a technique yielding delicate detail and subtle shading is essential. Papanicolaou's technique† and stain is eminently satisfactory in this regard.

Much of the clinical work on SR has been based on a critical level of 10 per cent; *i.e.*, if the patient has 9 per cent SR or less, she is said to have poor SR; if 10 per cent or more SR, she is said to have marked SR. Thus the cases can be placed into two groups. This particular dividing line has been arrived at empirically.

Cancer of the uterine cervix is commonly accompanied by marked SR. About 30 per cent of all cases with invasive cancers have SR. As may be seen in TABLE 1, stage I cases have 22 per cent SR; stage II, 37 per cent; stage III, 32 per cent; and stage IV, 18 per cent. Carcinoma *in situ* of that organ has associated SR in only 7 per cent of cases.

This rise and decline with the advancing stage of disease is regarded as the product of two forces. The lesion must reach a sufficient volume physically and metabolically to elicit a response initially and, under the deleterious influence of the advancing tumor, it is subsequently suppressed. It is probable that some patients never develop the sensitization response at all.

Cancers of other sites can elicit SR in the vaginal smear, some located close by and others more distant. Cancer of the uterine corpus is accompanied by marked SR in 35 per cent of the cases, cancer of the breast in 11 per cent. Nongenital cancers—for example, pancreas, stomach, and brain—are occasionally associated with SR in the vaginal smear. From time to time, SR is observed in the vaginal smear in the absence of demonstrable cancer. This occurs infrequently but is pronounced enough to be recorded in about 0.2 per cent of 28,000 negative vaginal smear cases. This figure lacks the precision of the previous ones, for it is not based on differential count. Nevertheless, it indicates the infrequency of SR in the absence of malignancy.

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* The work described in this paper was supported in part by a research grant (C-1810) from the Nationa Cancer Institute, National Institutes of Health, United States Public Health Service, Bethesda, Md.

† Immediate fixation while moist, in equal parts of 95 per cent alcohol and ethyl ether. The polychrome stain is commercially available as Ortho's EA-50 and OG-6.

TABLE 1

THE INCIDENCE OF MARKED SR IN THE VAGINAL SMEAR IN VARIOUS STATES

	Total number of cases examined	Number with marked SR
Cancer of cervix		
Stage 0	68	5 (7%)
Stage I	133	29 (22%)
Stage II	169	63 (37%)
Stage III	44	14 (32%)
Stage IV	11	2 (18%)
All invasive stages	357	108 (30%)
Cancer of uterine corpus	120	42 (35%)
Cancer of breast	36	4 (11%)
No cancer	28,300	64 (0.2%)

Cancers can elicit SR in adjacent squamous epithelium. In bronchogenic carcinoma, for example, it is seen in the sputum and, in carcinoma of the esophagus, in the gastric aspirate.

The initial significance of SR was prognostic for, when it was marked in cancer of the cervix, the patients were found to respond favorably to radiotherapy, the tumor and the patient apparently being sensitized to radiation; hence, the term "sensitization response." As may be seen in TABLE 2, the five-year results in patients treated radiologically are strikingly different when segregated according to SR.[1] Those with marked SR have a cure rate of 73 per cent; those with poor SR, a cure rate of 18 per cent. These differences appear at all levels, but are especially apparent in stages II and III.

It subsequently became evident that the same prognostic arrangement did not apply to patients treated surgically. Poor SR in the surgical case was not a bad sign. Indeed, on the contrary, it was a good omen in the clinically early case. In patients with stages I and II lesions, those with poor SR have a 74 per cent cure rate following surgery and a 24 per cent cure rate following radiation. Those with marked SR have a 43 per cent surgical cure rate and a 74 per cent radiotherapy cure rate. Other things being equal, one would apply

TABLE 2*

SR IN PATIENTS WITH CANCER OF THE CERVIX TREATED RADIOLOGICALLY: FIVE-YEAR RESULTS

	10% or more SR		9% or less SR	
	No.	Cure	No.	Cure
Stage I	12	7	23	6
Stage II	30	24	35	8
Stage III	8	6	20	1
Stage IV	1	0	7	0
All	51	37 (73%)	85	15 (18%)

* From Surg. Gynecol. & Obstet. 1954. **99:** 555.

TABLE 3

THE RELATION OF SR TO CURE IN CASES TREATED RADIOLOGICALLY OR SURGICALLY AND TO THE INCIDENCE OF REGIONAL METASTASES

Stage I and II Cancer of Cervix

	Radiated cases		Surgical cases		Regional metastases	
	No.	Cure*	No.	Cure†	No.	Positive Nodes
9% or less SR	58	14 (24%)	97	72 (74%)	113	18 (16%)
10% or more SR	42	31 (74%)	28	12 (43%)	33	20 (61%)

* Five year results.
† Two- to five-year results.

radiotherapy unquestionably to those with marked SR, and surgery to those with poor SR in stages I and II (TABLE 3).

A partial explanation for this prognostic schism lies in the frequency of regional metastases, for positive nodes seldom occur with poor SR (16 per cent) and are found rather frequently with marked SR (61 per cent). The five-year surgical cure rate is about 21 per cent in the face of positive nodes in cancer of the cervix, and ranges from 60 to 90 per cent when the regional lymph nodes are free of disease. Thus, poor SR in these early cases tends to include few cases with regional metastases, a circumstance that favors the surgical results. The obverse leads to the inescapable conclusion that in those with marked SR, in stages I and II, radiotherapy must cure a not inconsiderable number who have regional metastases.

The nature of SR has become apparent only slowly, and the picture is still incomplete, but this much is known:

(1) SR is a phenomenon of nonmalignant tissue. The cells that exhibit it are unmistakably benign.

(2) SR is observed in the normal epithelium near the cancer, but rarely at a distance. For example, marked SR was found in the pharyngeal smear of a young woman with carcinoma of the pharynx, but no SR was to be seen in the vaginal smear taken simultaneously.

(3) SR is enhanced by measures that improve the patient's general condition for, if the patient is kept in the hospital with its enforced rest, a high protein diet, and correction of anemia, the level of SR tends to rise.

(4) The administration of alpha tocopherol or testosterone propionate also may be followed by a rise in SR.

(5) Local trauma, such as the taking of a generous biopsy, may reduce the level of SR.

(6) Regional metastases tend to be directly related to SR. Marked SR is accompanied by frequent metastases; poor SR, by few.

On further inspection, one finds that the correlation between SR and positive nodes is bridged by the rate of desquamation of malignant cells. The shedding of cancer cells can be estimated by counting 100 consecutive epithelial cells differentially. The number of these found to be malignant is expressed as a percentage. There is great variation in the percentage of malignant cells observed in the vaginal smear, and the proportion bears no relation to the size of the tumor. The percentage may vary from less than 1 per cent to more than 90 per cent of all cells seen.

The incidence of desquamated malignant cells and SR exhibit a direct correlation. In stages I and II cancer of the cervix, only 15 per cent (14 of 94) of those with 9 per cent or less malignant cells show marked SR; 44 per cent (29 of 66) of those with 10 to 49 per cent malignant cells show marked SR; while 70 per cent (19 of 27) of those with 50 per cent or more malignant cells in the vaginal smear have marked SR.

In a group of 89 cases, only 2 (11 per cent) out of 18 with fewer than 1 per cent malignant cells in the smear had positive nodes, 12 (29 per cent) out of 41 with 9 per cent or less malignant cells had positive nodes; 7 (32 per cent) out of 22 with 10 to 49 per cent malignant cells had regional metastases; while 5 (63 per cent) out of 8, with more than 50 per cent malignant cells, had regional metastases. Thus it seems that, under host pressure, as indicated by SR, the tumor cells lose some of their adhesiveness and tend to fall off the main tumor mass and float away. Certainly this is true of the vaginal tumor, and the same probably holds true of the tumor surface in tissue and lymphatic spaces. Abundant scattering of seed is more likely to be followed by growth, even though the soil may be poor.

(7) SR is best produced in animals by foreign protein. If given intravenously, the response can be obtained, but repeated instillation in the vagina gives the best results. Horse serum instilled in the rabbit vagina three times a week for 2 to 5 weeks will result in magnificent SR. At least a week is required to produce this effect in the untreated animal. In the previously sensitized animal, however, the maximum response follows a single instillation in two to four days. SR tends to fall off unless the instillations are continued.

Oakley *et al.*[2] and others[3, 4] have demonstrated that antibodies can be produced in skin, muscle, mucous membrane, and other tissues. With this in mind, the above observation on foreign protein and SR takes on the appearance of an immune response.

(8) The cytoplasm of the SR cell absorbs markedly in the ultraviolet.* The absorption pattern is consistent with that of gamma globulin.

(9) The prognostic significance of SR in the patients treated radiologically has been reviewed above.

SR has come to be regarded as a local manifestation of resistance to the tumor, probably a production of antibody in the cells in response to a tumor antigen.

There are two major factors determining SR: the antigenicity of the tumor, and the reactivity of the patient. If the tumor is highly antigenic and sufficiently abundant, SR tends to be marked. If, however, the tumor chemically resembles the host so closely that it is nonantigenic, or the tumor itself is too small, or the host unresponsive, no SR will be elicited. An entire spectrum of cases resulting from these two elements can be distinguished. The typical case that begins as a carcinoma *in situ* and gradually develops into a stage I lesion has no SR and it desquamates few cells. In these stages, surgery is the treatment of choice. Later, if unchecked, the bulk of the tumor develops so much that a response is elicited, marked SR is found and, under host pressure, cells desquamate freely and metastasis begins. Radiotherapy here is especially effective. Later, if the tumor is not discovered, gradual progression of the tumor undermines the patient's defenses, resistance deteriorates, and SR is lost. This is the more advanced case in which either radiotherapy and surgery offers scant hope of cure. It is this same situation that may respond to the administration of alpha tocopherol or testosterone propionate by restoring the patient's reactivity and making it possible for her to respond effectively to irradiation. Of course, the point is ultimately reached when restoration is impossible and radiation is of no avail. There are other cases in whom SR either never develops because the tumor is antigenically inert or the patient is insensitive. These patients, if treatment is to be successful, must have the tumor excised.

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* Observed on the Polaroid Color-Translating Ultraviolet Microscope through the courtesy of the American Cancer Society, New York, N. Y.

Clinical, cytologic, and histologic experience suggests that underlying host immune and inflammatory reaction against the tumor is essential if radiotherapy is to produce the desired effect. In animals, it has been shown that radiotherapy is most effective in controlling the tumor when the spontaneous host resistance to the tumor is maximum, and least effective when it is lowest.[5, 6]

Conclusion

The sensitization response represents a potent host reaction to the tumor that influences the growth and spread of the cancer, as well as its course following treatment. Armed with the better understanding provided by SR, improved cancer control may be possible.

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